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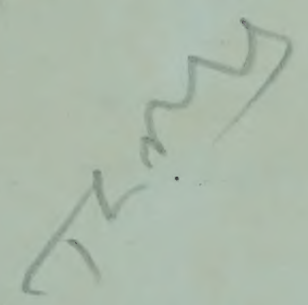
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Symposium on ami.







- 3256
- ① amino acid metabolism
  - ② amino acids
  - ③ amino acid transport
  - ④ enzyme formation
  - ⑤ adaptive enzymes
  - ⑥ proteins
  - ⑦ peptide biosynthesis
  - ⑧ pantothenic acid
- 
- 







*A Symposium on*

# AMINO ACID METABOLISM

*Sponsored by*  
THE  
McCOLLUM-PRATT INSTITUTE  
OF  
THE JOHNS HOPKINS UNIVERSITY

*Edited by*  
WILLIAM D. McELROY AND H. BENTLEY GLASS



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## Part I

### *GENERAL CONSIDERATION OF AMINO ACID METABOLISM*





# GENERAL REACTIONS OF AMINO ACIDS

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THE METABOLISM OF amino acids may be considered in terms of at least three types of reactions: (1) reactions leading to the synthesis of amino acids; (2) reactions which result in the combination of amino acids to form protein; and (3) reactions which involve the conversion of amino acids to other products, e. g., (a) formation of urea, uric acid, allantoin, creatine, and products which also arise in the course of the metabolism of fats and carbohydrates; (b) conversion of one amino acid to another; and (c) utilization of amino acids for the synthesis of peptides (e. g., glutathione, carnosine), vitamins (e. g., pantothenic acid, folic acid, nicotinic acid), and many other compounds (e. g., melanin, porphyrin, nucleic acid, histamine).

In 1912, Kossel (1), referring to the problem of the structure of proteins, stated: "We can obtain some idea of the possible variety in the combinations of the protein Bausteine by recalling the fact that they are as numerous as the letters in the alphabet which are capable of expressing an infinite number of thoughts." The metabolic versatility of the amino acids, and the fact that more than 50 amino acids are now known to occur in nature, suggests that this statement may be applicable also to the metabolic transformations of amino acids.

Although it is obvious that the available information on the biological reactions of amino acids cannot be expressed exclusively in terms of a few generalizations, there are at least several categories of reactions in which a number of amino acids participate, and which may therefore be considered as "general reactions." These include:

## (1) Deamination



- (2) Decarboxylation
- (3) Transamination
- (4) Peptide and protein synthesis

It is anticipated that the formidable problem of protein synthesis will be considered by other speakers at this symposium. The present discussion will therefore be limited mainly to recent developments concerning the first three of these topics. It is necessary to treat only cursorily some aspects of these fields, and it is expected that these and related subjects will receive additional attention during the discussion.

### DEAMINATION

#### *D-Amino Acid Oxidase.*

The first definitive enzymatic studies on the deamination of amino acids were carried out by Krebs (2, 3), who observed oxidation of both D- and L-amino acids by kidney and liver preparations. These reactions were shown to be catalyzed by separate optically specific enzyme systems. The D-amino acid oxidase of kidney has been purified, extensively investigated (4), and shown to require flavin adenine dinucleotide as a coenzyme (5). The oxidation may be considered to take place in accordance with three reactions, of which only the first is enzymatic:



As yet, unequivocal evidence for the formation of the intermediate imino acid has not been obtained. In the presence of catalase, reaction (3) does not take place, and the products include the analogous  $\alpha$ -keto acid and ammonia. A large body of evidence regarding the specificity of D-amino acid oxidase has accumulated,

and in addition to the liver and kidney of many animals, the enzyme has been found in certain molds (6, 7) and bacteria (8, 9).

#### *L-Amino Acid Oxidase.*

It is curious that although certain animal tissues are equipped with active enzymes which catalyze the oxidation of D-amino acids, the L-amino acid oxidase activity of mammalian tissues is relatively low. Blanchard et al. (10) described a purified rat kidney L-amino acid oxidase possessing riboflavin phosphate as a coenzyme, and catalyzing the oxidation of 13 L-amino acids as well as certain L- $\alpha$ -hydroxy acids. The low activity of this system, and its limited distribution, suggest that it may be of only minor significance in the deamination of L-amino acids. In the light of recent developments in transamination (see below), it appears probable that the deamination of L-amino acids takes place in mammalian tissues by a mechanism involving transamination with  $\alpha$ -ketoglutarate, and deamination of the resulting glutamate by glutamic dehydrogenase.

In contrast to the weak activity of L-amino acid oxidase in tissues of mammals, the venom and tissues of certain snakes exhibit considerable L-amino acid oxidase activity (11, 12). Purified ophio-amino acid oxidase has been shown to require flavin adenine dinucleotide as a coenzyme (13). A number of molds (14, 16) and bacteria (17) also possess L-amino acid oxidase activity. The mechanism of oxidation described above (equations 1 to 3) is compatible also with the experimental observations for L-amino acid oxidase.

#### *Specificity of the Amino Acid Oxidases.*

Representative data taken from the literature on the relative rates of oxidation of amino acids by several oxidase preparations are given in Table 1. Kidney D-amino acid oxidase does not oxidize substrates in which the  $\alpha$ -hydrogen atom or both amino group hydrogen atoms are substituted. Certain N-monomethyl-D-amino acids and D-proline are susceptible. Ophio-L-amino acid oxidase does not act on N-methyl-L-amino acids or L-proline, and also requires an unsubstituted  $\alpha$ -hydrogen atom. Neither the D- nor the L-oxidase attacks  $\beta, \beta, \beta$ -trimethylalanine (*tert.* leucine), while both



TABLE 1  
SPECIFICITY OF SEVERAL AMINO ACID OXIDASES

Amino Acid	L-Oxidase ( <i>N. crassa</i> ) <sup>1</sup>	L-Oxidase (Rattlesnake venom) <sup>2</sup>	L-Oxidase (Rat Kidney) <sup>3</sup>	D-Oxidase (Sheep Kidney) <sup>1</sup>
Alanine	53	0.4	—	34
$\alpha$ -Aminoadipic acid	78	7	—	0
$\alpha$ -Aminobutyric acid	82	20	3	16
Arginine	—	7	—	—
Aspartic acid	6	<0.1	0	0.5
Cystine	72	26	15	1
Glutamic acid	12	<0.1	0	0
Histidine	47	14	9	3
Isoleucine	42	29	71	12
Leucine	(100) <sup>4</sup>	92	(100) <sup>4</sup>	7
Lysine	18	0.2	0	0.3
Methionine	51	(100) <sup>4</sup>	81	42
Ornithine	65	0.1	0	2
Phenylalanine	53	76	45	14
Proline	0	0	77	78
Serine	10	0	0	22
Threonine	3	0	0	1
Tryptophan	35	82	40	19
Tyrosine	31	76	20	(100) <sup>4</sup>
Valine	8	4	28	18

<sup>1</sup> Data of Bender and Krebs (14)

<sup>2</sup> Data of Greenstein et al. (18)

<sup>3</sup> Data of Blanchard et al. (10)

<sup>4</sup> Activity arbitrarily assigned a value of 100; other values given as percentage of rate with most active substrate.

enzymes oxidize the respective isomers of  $\alpha$ -phenylglycine. Thus, the insusceptibility of *tert.* leucine may probably be ascribed to steric hindrance, rather than to the absence of a  $\beta$ -hydrogen atom. Oxidation of the four isomers of isoleucine results in formation of the corresponding optically active  $\alpha$ -keto acids, a fact indicating that enzymatic oxidation does not involve  $\alpha$ - $\beta$  unsaturation leading to loss of configuration at the  $\beta$ -carbon atom (19). Similar findings were made by Fones (20), who reported that enzymatic oxidation of the *threo* and *erythro* isomers of phenylserine yielded optically active mandelic acids.

As indicated in Table 1, the relative susceptibility of amino acids to oxidation varies considerably with different enzymes. A large number of other amino acids are oxidized by these or similar enzyme preparations, including citrulline, glutamine, homoserine, asparagine,

norvaline, norleucine, and a good many others. (See, e. g., 3, 11, 12, 14, 18, 21, 22).

The existence of separate oxidases for certain amino acids has been reported, e. g., D-aspartic acid (23), L-cysteine (24, 25), glycine, and sarcosine (26). The significant role of glutamic dehydrogenase in the deamination of amino acids will be considered below.

#### *Possible Metabolic Functions of the Amino Acid Oxidases.*

The function of D-amino acid oxidase in animal tissues is unknown. It has been suggested that it may destroy D-amino acids formed by symmetrical amino acid synthesis. In this connection, it may be said that there is as yet no evidence for symmetrical amino acid synthesis in animals, and there is good evidence against it. Shemin and Rittenberg found that animals fed  $N^{15}$ -labelled DL-glutamate and DL-tyrosine excreted urinary D-tyrosine and D-glutamate which showed no appreciable dilution of  $N^{15}$  (27).

It is now known that a number of D-amino acids occur in nature, and that in certain cases they may be formed by the action of racemases. The function of D-amino acid oxidase may be to destroy such D-amino acids as may be ingested by an animal or be formed "accidentally" by a cell. Thus, D-amino acid oxidase would protect against the accumulation of certain D-amino acids, which might otherwise serve as substrates for less optically specific enzyme systems. It must be noted, however, that D-glutamic acid, which is known to occur naturally, is a very poor substrate for D-amino acid oxidase. Compatible with this teleological approach to the role of D-amino acid oxidase is the ability of animals and some bacteria to utilize D-amino acids in place of their enantiomorphs, findings which may probably be ascribed to the consecutive action of D-amino acid oxidase and L-specific transaminase.

There are a number of other enzymatic reactions in which D-amino acids have been found to participate, e. g., desulfuration of D-cysteine (28, 29), dehydration of D-serine (30, 31), hydrolysis of D-peptides (32), synthesis of D-glutamine (33), and hydrolysis of D-asparagine (34). The superiority of D-alanine over its enantiomorph in supporting the growth of *Lactobacillus casei* and *Streptococcus fecalis*



suggests that this D-isomer may have a significant function in the metabolism of these organisms (35-37). Preliminary reports have appeared describing the participation of D-glutamic acid and D-glutamine in transpeptidation reactions (38), and of D-alanine and D-glutamic acid in transamination (39) in preparations of *Bacillus subtilis*, an organism which synthesizes polyglutamic acid predominantly of the D-configuration.

Although there is little evidence that the L-amino acid oxidase of the tissues of higher animals plays a significant part in amino acid metabolism, the very active L-oxidase of certain snakes and microorganisms may be of metabolic importance in these organisms. Coupled oxidation-reduction reactions of the "Stickland" type are of significance in certain anaerobic organisms, and these reactions have been reviewed recently by Nisman (40).

#### *Some Practical Values of Amino Acid Oxidase.*

Although it has thus far been somewhat difficult to establish their metabolic function, the value of the amino acid oxidases as analytical and preparative tools in the study of amino acids and their metabolism has been amply demonstrated. The strict antipodal specificity of kidney D-amino acid oxidase and of ophio-L-amino acid oxidase makes it possible to determine quantitatively (by following oxygen uptake or ammonia formation) many amino acid isomers in the presence of considerable concentrations of their enantiomorphs. The optical purity of a number of amino acid preparations may be determined to 1 part in 1000, and in at least two instances to 1 part in 10,000, representing a degree of sensitivity not available by polarimetric measurement (41). Preparation of D- or L-isomers of an amino acid from the corresponding racemate may be accomplished by selective destruction of one isomer by using the appropriate oxidase. Finally, the oxidases may be employed for the preparation of  $\alpha$ -keto acids. After oxidation of the susceptible isomer, the keto acid may be isolated in pure form by convenient procedures (42).

## DECARBOXYLATION

A number of animal tissues, bacteria, and plants possess enzymes which catalyze the decarboxylation of L-amino acids according to the following equation:



The studies of Werle, Gale, Blaschko, and Schales have established many of these reactions and elucidated the catalytic systems involved; these important contributions have been recently reviewed (43-46).

TABLE 2  
AMINO ACID DECARBOXYLATION REACTIONS

Amino Acid	Product	Source	References
Arginine*	Agmatine	B	(46a)
Aspartic Acid	$\beta$ -Alanine	B	(47)
Aspartic Acid *	L-Alanine	B	(48, 49)
Cysteic Acid	Taurine	A	(50)
$\alpha,\epsilon$ -Diaminopimelic Acid*	L-Lysine	B	(51)
3,4-Dihydroxyphenylalanine*†	3,4-Dihydroxyphenylethylamine	A, B	(46, 46a, 52, 53)
Glutamic Acid*	$\gamma$ -Aminobutyric Acid	A, B, P	(44, 46, 54-57)
Histidine	Histamine	A, B	(45, 58)
$\beta$ -Hydroxyglutamic Acid*	$\gamma$ -Amino- $\beta$ -hydroxybutyric Acid	B	(59)
5-Hydroxylysine	(Hydroxycadaverine)‡	B	(60)
<i>p</i> -Hydroxyphenylserine	( <i>p</i> -Hydroxyphenylethanolamine)‡	A	(43)
5-Hydroxytryptophan*	5-Hydroxytryptamine	A	(61)
Lysine*	Cadaverine	B	(44, 62)
$\gamma$ -Methyleneglutamic Acid	$\gamma$ -Amino- $\alpha$ -methylenebutyric Acid	P	(63)
Ornithine*	Putrescine	B	(44, 46a)
Phenylalanine*	Phenylethylamine	B	(64)
Tyrosine*	Tyramine	A, B	(44-46a)

A = mammalian tissue; P = plant tissues; B = microorganisms

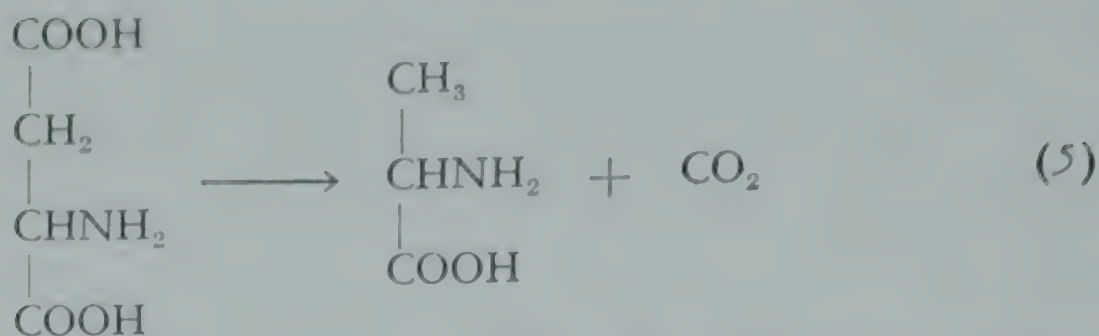
\* Activation by pyridoxal phosphate reported

‡ Identification not yet reported

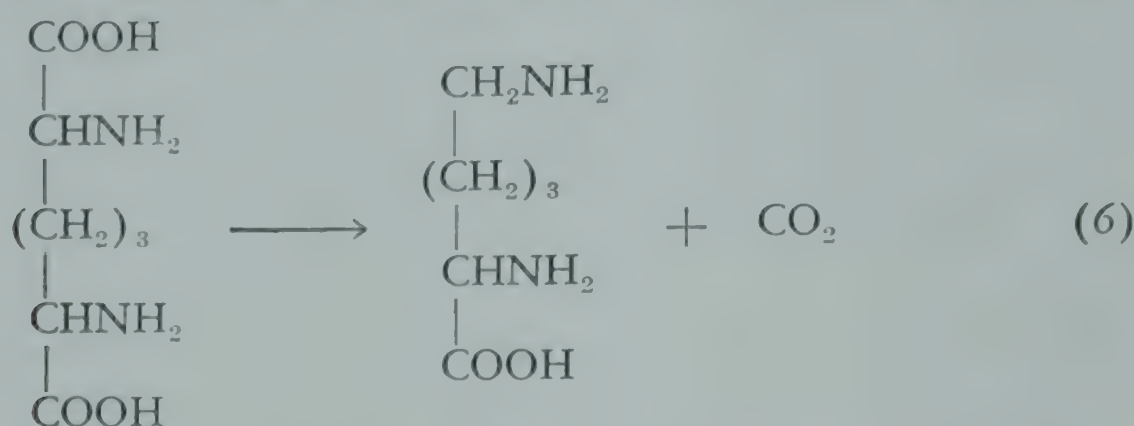
† Certain other hydroxy derivatives of phenylalanine are also decarboxylated.

The decarboxylase reactions which have thus far been described are summarized in Table 2. Among the more recently discovered decarboxylase systems is the L-aspartic acid  $\beta$ -decarboxylase of *Clostridium welchii* (48), which is unique in that the  $\beta$  rather than the  $\alpha$ -carboxyl is attacked:

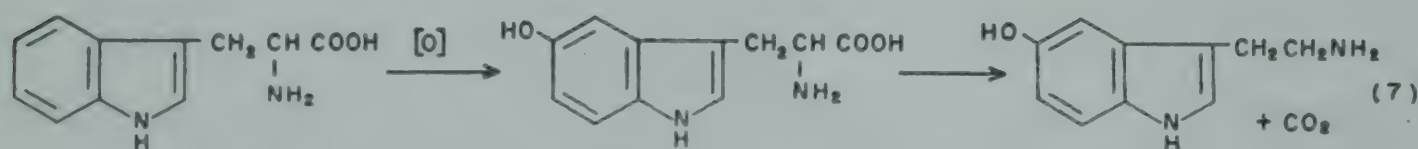




Another reaction which yields an  $\alpha$ -amino acid is the decarboxylation of  $\alpha,\epsilon$ -diaminopimelic acid to L-lysine (51),<sup>1</sup> a reaction of importance in the formation of lysine in *Escherichia coli* (65):

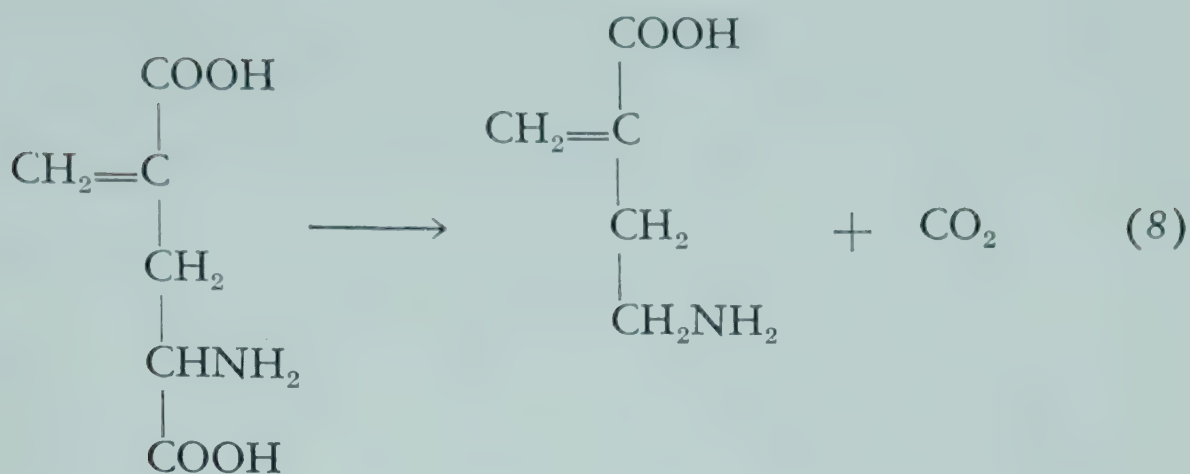


The decarboxylation of tryptophan has been reinvestigated by Udenfriend, Clark, and Titus (61), who found that this reaction did not occur in mammalian tissues. The major pathway for decarboxylation now appears to involve conversion of tryptophan to 5-hydroxytryptophan, which is decarboxylated to 5-hydroxytryptamine (serotonin):



Done and Fowden (66) have reported the isolation of  $\gamma$ -methylene-glutamic acid from the peanut plant, and the presence of this amino acid in the tulip bulb has been demonstrated by Zacharias, Pollard, and Steward (67). Fowden and Done (63) subsequently isolated  $\gamma$ -amino- $\alpha$ -methylenebutyric acid from the peanut plant, and showed that extracts of the latter plant, as well as of red pepper and barley roots, catalyzed the decarboxylation of  $\gamma$ -methyleneglutamic acid to  $\gamma$ -amino- $\alpha$ -methylenebutyric acid:

<sup>1</sup> See Work, E., page 462, this volume.



Umbreit and Heneage (59) have described the decarboxylation of one of the optical isomers of *allo*- $\beta$ -hydroxy-DL-glutamic acid by preparations of several bacteria. It is of interest that  $\beta$ -hydroxy-DL-glutamic acid was not attacked.  $\gamma$ -Amino- $\beta$ -hydroxybutyric acid was formed, and the evidence suggested that the decarboxylase was not identical with glutamic decarboxylase. At this time no evidence exists for the natural occurrence of a  $\beta$ -hydroxyglutamic acid (68).

The decarboxylation of valine and leucine to the corresponding amines by *Proteus vulgaris* cells has been described in a preliminary report (69).

The role of pyridoxal phosphate as a coenzyme for the decarboxylation of amino acids in plants and animal tissues, and in microorganisms, has been demonstrated in almost all instances in which this coenzyme has been studied, although apparently histidine decarboxylase may be an exception in this respect (cf. 70).

Decarboxylase reactions in animal tissues are of significance in the formation of certain compounds such as taurine, histamine, serotonin, etc. The formation of  $\gamma$ -aminobutyric acid by decarboxylation of glutamic acid may be of importance in the metabolism of brain. The role of amino acid decarboxylation in bacterial metabolism has been discussed by Gale (71), and by Gunsalus (72). Present evidence indicates that some decarboxylation reactions are not appreciably reversible. However, reversibility is not by any means excluded in all decarboxylase reactions, and it is possible that these enzymes may catalyze the synthesis of certain amino acids from carbon dioxide and the corresponding amines.

Finally, it should be mentioned that the use of bacterial decar-



boxylases for the quantitative estimation of amino acids (44) represents another example of a way in which the study of amino acid metabolism has contributed to its own technical development.

### TRANSAMINATION <sup>2</sup>

The classical experiments of Neubauer (74) and of Knoop (75) demonstrated the conversion of amino acids to  $\alpha$ -keto acids in several animal species, and the reverse reaction, viz., amination of an  $\alpha$ -keto acid, was also shown. The discovery of the enzymatic transamination reaction by Braunstein and Kritsmann (76) in 1937 represented an important contribution to the understanding of these metabolic transformations. Until several years ago, it was generally believed that transamination was mainly limited to reactions involving aspartate, glutamate, alanine, and their  $\alpha$ -keto analogues (77-79). Studies carried out in the last few years, in which more sensitive analytical methods were used, have led to the conclusion that a large number of L-amino acids participate in transamination reactions. This concept is consistent with certain earlier studies on transamination (78, 79), with observations on the rapid incorporation of administered amino acid nitrogen into almost all of the amino acids of animals (80), and with the ability of animals and microorganisms to utilize the  $\alpha$ -keto analogues of some amino acids for growth.

Rapid progress in our knowledge of transamination in recent years has been made possible by the application of better techniques of amino acid identification and quantitation (for example, by means of paper chromatography, and of specific amino acid decarboxylases), and also by the development of improved and convenient procedures for the preparation of the optical isomers (81) and  $\alpha$ -keto analogues (82) of most of the naturally-occurring amino acids.

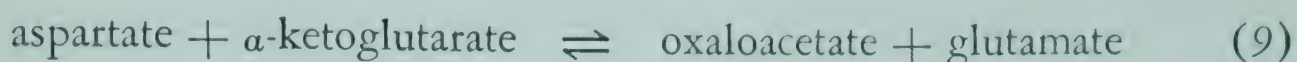
#### *Reactions Between $\alpha$ -Ketoglutarate and Amino Acids.*

Early studies clearly demonstrated the occurrence of the glutamate-aspartate and glutamate-alanine reactions, and that these were catalyzed by separate enzymes of relatively high specificity. The aspar-

<sup>2</sup> Time does not permit an exhaustive review of this subject. A more complete discussion of transamination is given elsewhere (73).



tate-alanine reaction, at least in heart muscle, may be attributed to the combined action of the two glutamate transaminases (83, 84):



It is now apparent from a number of investigations that many other  $\alpha$ -ketoglutarate-amino acid reactions are catalyzed by preparations of animal tissues and microorganisms (e. g., see 85-90). The results of some of these studies are summarized below.

Feldman and Gunsalus (85) reported that dried cells of several bacterial species catalyzed the formation of glutamate from  $\alpha$ -ketoglutarate and the following amino acids: aspartate, alanine, valine, leucine, norleucine, tryptophan, tyrosine, phenylalanine, and methionine. Somewhat less activity was observed with isoleucine, histidine, lysine, glycine, and threonine. These authors also demonstrated the reversibility of the  $\alpha$ -ketoglutarate-phenylalanine and  $\alpha$ -ketoglutarate-tyrosine reactions.

Cammarata and Cohen (86) investigated the formation of glutamate from  $\alpha$ -ketoglutarate and amino acids in crude preparations of several animal tissues. They reported glutamate formation from aspartate, valine, leucine, isoleucine, alanine, tyrosine, phenylalanine, methionine, tryptophan, arginine, cysteine, serine, histidine, glycine, lysine, ornithine, diiodotyrosine, DOPA,  $\alpha$ -aminobutyric acid, taurine, and norleucine. The formation of glutamate was low with several of these.

Tanenbaum and Shemin (87) have reported the formation of  $\text{N}^{15}$ -glutamate from  $\alpha$ -ketoglutarate and  $\text{N}^{15}$ -leucine with a pig heart muscle preparation. The ammonia isolated in these experiments had a low isotope content, a finding consistent with the concept that glutamate was formed by a direct reaction between  $\alpha$ -ketoglutarate and leucine.

Transamination between  $\alpha$ -ketoglutarate and cysteic acid was described in early studies (78, 79), and recently Darling (91) has presented evidence suggesting that this reaction may be catalyzed by a separate enzyme. The formation of cysteic acid from sulfo-



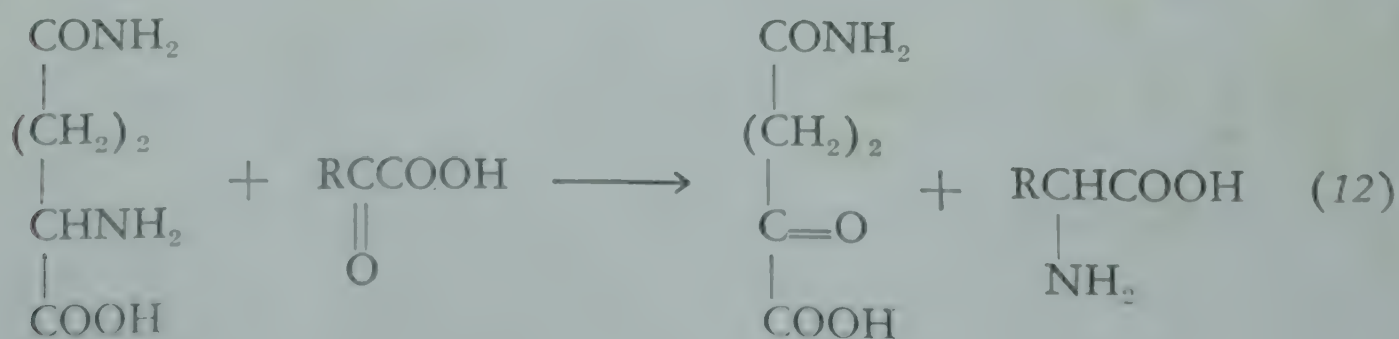
pyruvate by transamination with glutamine has also been described (92).

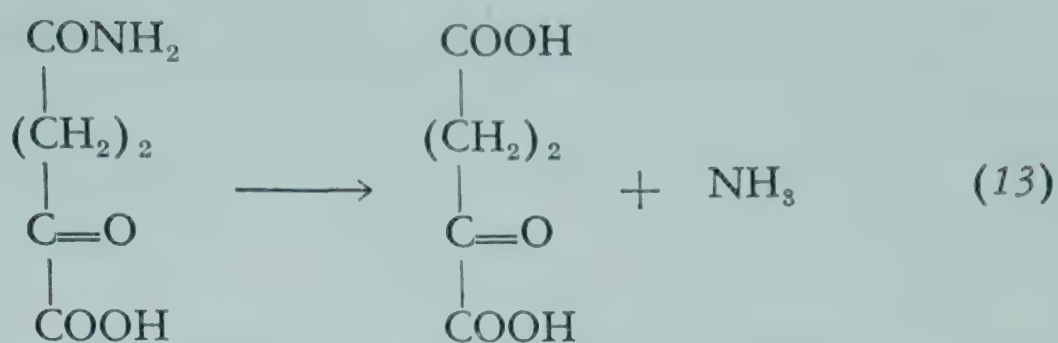
Transamination reactions between  $\alpha$ -ketoglutarate and isoleucine, catalyzed by preparations of hog heart and certain microorganisms, have been investigated (88). L-Alloisoleucine and its keto analogue (1- $\alpha$ -keto- $\beta$ -methylvalerate) transaminate at somewhat slower rates than L-isoleucine and d- $\alpha$ -keto- $\beta$ -methylvalerate. However, transamination (in either direction) does not result in loss of the configuration of the  $\beta$ -carbon atom, an observation analogous to those made on the enzymatic oxidation of the four isomers of isoleucine (see above).

Fowden and Done (92a) have reported that two derivatives of glutamic acid,  $\gamma$ -methylglutamic and  $\gamma$ -methyleneglutamic acids, are capable of transaminating with  $\alpha$ -ketoglutarate, oxaloacetate, and pyruvate in extracts of the peanut plant.

#### *Transamination Involving Glutamine and Asparagine.*

Meister and Tice (93) have reported transamination catalyzed by liver preparations between glutamine and a number of  $\alpha$ -keto acids, and resulting in the formation of  $\alpha$ -ketoglutarate, ammonia, and the corresponding amino acids. This reaction was first recognized by Greenstein and Carter (94), who observed the accelerating effect of pyruvate on the deamidation of glutamine in rat liver extracts. Recent evidence (93, 95) suggests that transamination occurs prior to deamidation, and the observation that the enzyme preparation catalyzes the hydrolysis of  $\alpha$ -ketoglutaramic acid to ammonia and  $\alpha$ -ketoglutarate is compatible with the concept that the  $\alpha$ -keto acid- $\omega$ -amide is formed by transamination, and subsequently split by a specific amidase:





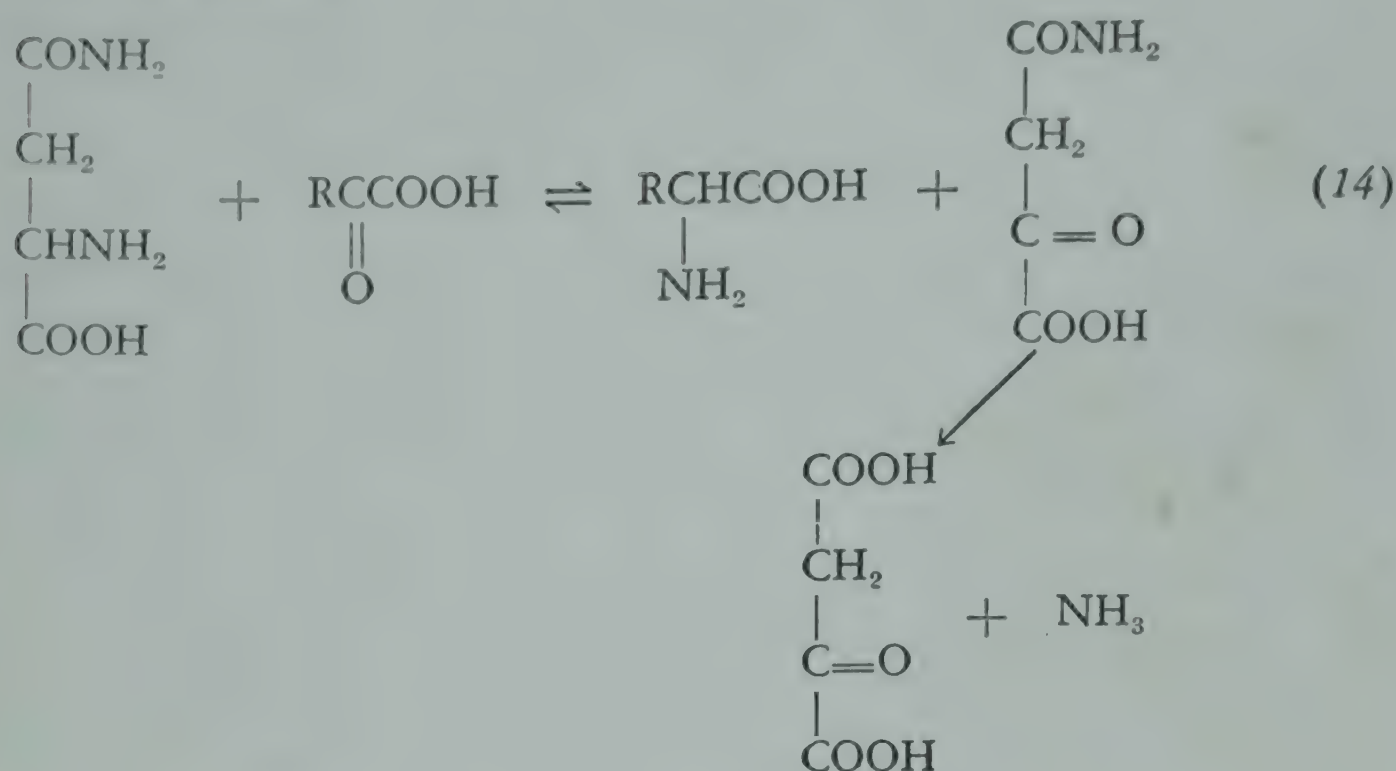
Further support for this reaction sequence arose from studies with two glutamine derivatives ( $\gamma$ -methylglutamine and  $\gamma$ -methyleneglutamine), which participated in transamination, but which were not deamidated in the course of the reaction (96). In the  $\gamma$ -methylglutamine-keto acid reaction, formation of  $\alpha$ -keto- $\gamma$  methylglutaramic acid was demonstrated, and preparations of this keto acid amide were found to be insusceptible to the action of the amidase. Although the amidase activity, which is considerably greater than that of the transaminase, was isolated free of transaminase activity and purified approximately 60-fold, the transaminase has not yet been obtained free of amidase activity.

The broad specificity of the transaminase system is indicated by the fact that more than 30  $\alpha$ -keto acids are active, including the  $\alpha$ -keto analogues of the following amino acids (92, 93, 95-99): alanine, glycine, serine,  $\alpha$ -aminobutyric acid, norvaline, norleucine, leucine, phenylalanine, tyrosine, cyclohexylalanine,  $\alpha$ -aminocaprylic acid, methionine, ethionine, glutamate, tryptophan, asparagine, cysteine, arginine, and cysteic acid.  $\alpha$ -Keto acids possessing no or only one  $\beta$ -hydrogen atom, such as  $\alpha$ -ketoisovaleric,  $\alpha$ -keto- $\beta$ -methylvaleric, and trimethylpyruvic, and the  $\alpha$ -keto analogues of lysine and ornithine, which are known to cyclize (97), were inactive.

Other liver fractions catalyze analogous transamination reactions between asparagine and  $\alpha$ -keto acids (98), in which the products are ammonia, oxaloacetate, and the corresponding amino acids. The properties of this system are similar to those of the glutamine-keto acid system, and the enzyme preparation also deamidates  $\alpha$ -ketosuccinamate to oxaloacetate. In the presence of high concentrations of  $\alpha$ -ketosuccinamic acid and a wide variety of L-amino acids (analogous to the active  $\alpha$ -keto acids), L-asparagine formation was



observed (100). The asparagine- $\alpha$ -keto acid reaction may be represented as follows:



The possibility that this reaction may be involved in asparagine synthesis remains to be explored.

It is of interest that other reports have appeared describing the superiority of glutamine and asparagine over the respective  $\alpha$ -amino dicarboxylic acids in the transamination of glyoxylate (100a) and of phenylpyruvate and *p*-hydroxyphenylpyruvate (100b). Non-enzymatic transamination of glyoxylate to glycine also proceeds more rapidly with glutamine and asparagine than with glutamate or aspartate (138).

#### *Other Reactions Not Involving Dicarboxylic Amino or Keto Acids.*

In addition to the glutamine- and asparagine- $\alpha$ -keto acid reactions, a number of other transamination reactions not involving glutamate, aspartate, or their keto analogues have been recently described. Rowsell (101) reported evidence, based on qualitative paper chromatographic study, for the occurrence of amino acid-pyruvate transamination in liver preparations, in which  $\alpha$ -ketoglutarate, glutamate, and aspartate apparently did not participate.

Another reaction of this type was described by Quastel and Witty (102), who demonstrated transamination between ornithine and

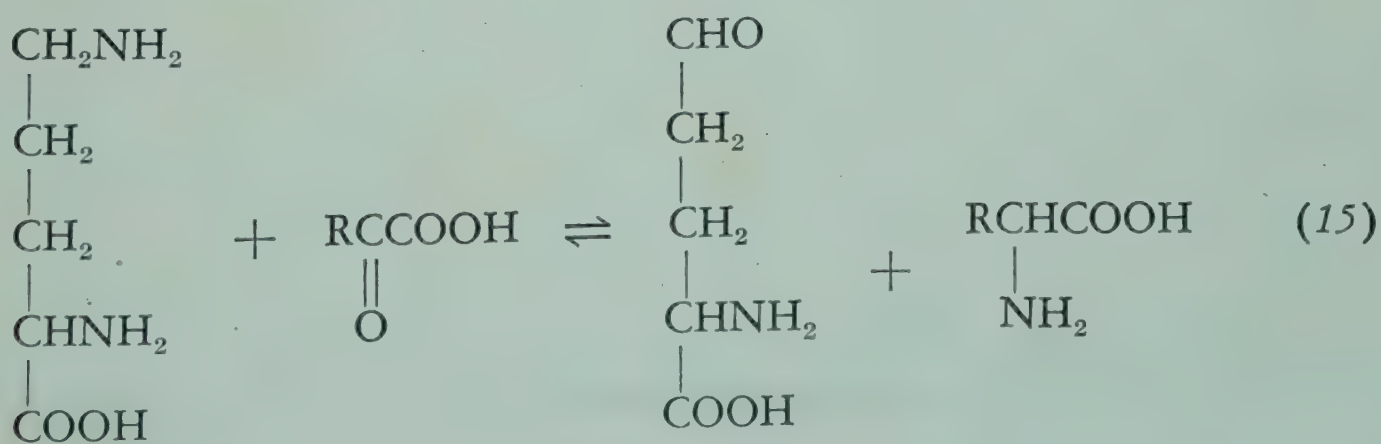
pyruvate catalyzed by kidney and liver preparations. The ornithine-keto acid reactions will be described below.

Rudman and Meister (89) reported several transamination reactions in *Escherichia coli* between monocarboxylic amino acids, and in one of these the participation of a dicarboxylic amino acid was excluded. The enzyme responsible for this reaction was completely separated from the glutamate transaminases and was shown to catalyze only reversible transamination between  $\alpha$ -ketoisovalerate and alanine,  $\alpha$ -aminobutyric acid, and, to a slight extent, norvaline. Glutamate, aspartate, a wide variety of other amino acids, and their  $\alpha$ -keto analogues were completely inactive.

Altenbern and Housewright (103) have reported that sonic extracts of *Brucella abortus* possess transaminases which catalyze certain pyruvate-amino acid reactions leading to alanine formation. The leucine-pyruvate reaction was studied in detail, and a partial separation of the leucine-glutamate and leucine-alanine systems was obtained.

#### *Transamination Involving $\omega$ -Amino Groups and Aldehydes.*

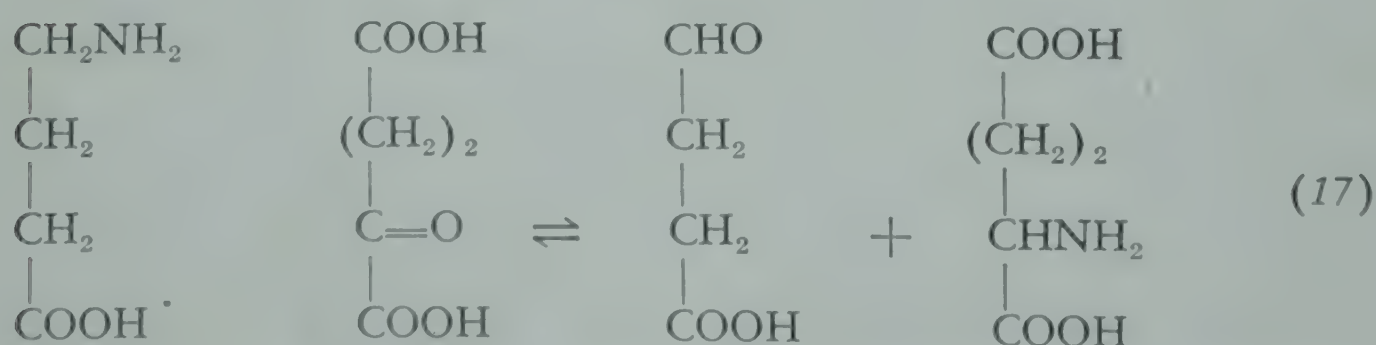
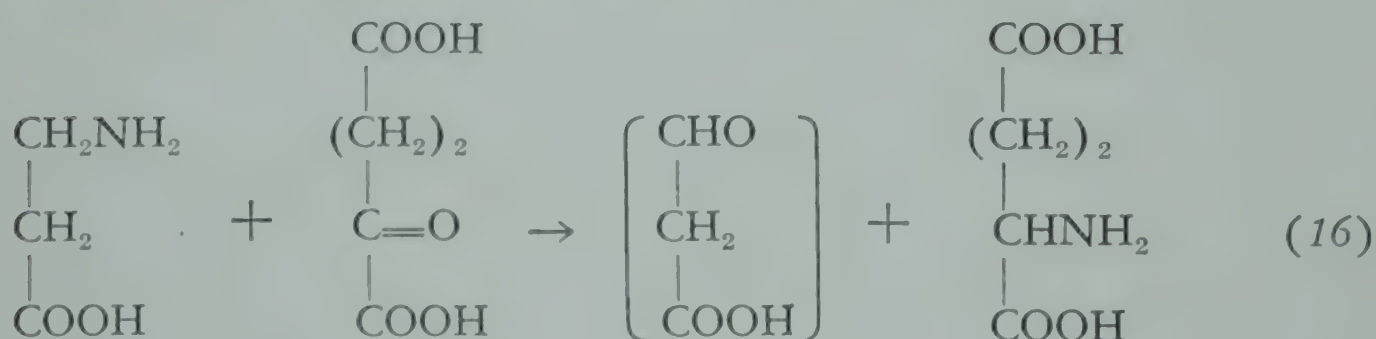
The observations of Quastel and Witty (102) on transamination between ornithine and pyruvate in animal tissues have been mentioned above. Fincham (104), in an investigation of proline and ornithine metabolism in *Neurospora crassa* observed that mycelial extracts catalyzed the formation of glutamate and glutamic- $\gamma$ -semi-aldehyde from ornithine and  $\alpha$ -ketoglutarate. In liver preparations, Meister (99) found that ornithine transaminated with pyruvate,  $\alpha$ -ketoglutarate,  $\alpha$ -ketobutyrate, glyoxylate, and several other keto acids to yield the corresponding amino acids and glutamic- $\gamma$ -semi-aldehyde:





Transamination between ornithine and glyoxylate represents a special case in which an aldehyde is both a reactant and a product. It is of great interest that the  $\delta$  rather than the  $\alpha$ -amino group of ornithine transaminates. Transamination of the  $\alpha$ -amino group has been observed when the  $\delta$ -amino group of ornithine or that of its  $\alpha$ -keto analogue is substituted (88, 99). Recently, Vogel (105) has reported that the pathway of ornithine synthesis in *E. coli* involves formation of N-acetylglutamic- $\gamma$ -semialdehyde, which is converted by transamination to  $\alpha$ -N-acetylornithine. It is also possible that the  $\omega$ -amino group of lysine may participate in transamination (see page 28).

Two additional examples of transamination involving  $\omega$ -amino groups have been described (106, 107). Bessman et al. (107) reported transamination between  $\gamma$ -aminobutyrate and  $\alpha$ -ketoglutarate in brain homogenates. The formation of succinic semialdehyde and of glutamate was demonstrated, and the reaction was found to be reversible. Roberts and Bregoff (106) confirmed the  $\gamma$ -aminobutyrate- $\alpha$ -ketoglutarate reaction, and showed that brain preparations could also catalyze transamination between  $\beta$ -alanine and  $\alpha$ -ketoglutarate. These reactions have also been observed with preparations of liver and of certain microorganisms (107a).



Transamination between glyoxylate and glutamine, glutamate, aspartate, and asparagine to yield glycine has been reported (108,

98). The reverse reaction leading to glyoxylate formation proceeds less readily.

*Evidence for the Existence of Separate Transaminases.*

Unfortunately only a few transaminases have thus far been purified, and even in these cases it is evident that a high state of homogeneity has not been achieved. There is, however, a great deal of evidence for the existence of a number of transaminases. The glutamate-aspartate and glutamate-alanine transaminases of heart muscle have been separated and purified (83, 109-112). The existence of a separate aspartate-alanine enzyme is in doubt, since aspartate-alanine activity can be accounted for by the combined action of the glutamate-alanine and glutamate-aspartate enzymes (83, 84). Kritsmann and Samarina (113) have reported the isolation of an aspartate-alanine enzyme from pigeon liver, a finding which Cammarata and Cohen (114) were unable to confirm.

In studies on *E. coli*, Rudman and Meister (89) obtained fractions which catalyzed transamination reactions between the amino acids (or keto analogues) of the following groups: (A) glutamate, aspartate, tryptophan, phenylalanine, and tyrosine; (B) glutamate and certain aliphatic amino acids; (C) valine and alanine (or  $\alpha$ -aminobutyric acid). As described in Table 3, fraction A exhibited

TABLE 3  
TRANSAMINATION BETWEEN AMINO ACIDS AND  $\alpha$ -KETOGLUTARATE  
CATALYZED BY *E. coli* PREPARATIONS \*

L-Amino Acid	Enzyme Preparation			
	Wild Strain Extract	Fraction A	Fraction B	Mutant Extract
Isoleucine	16.4	0	210	0
Valine	12.5	0	145	0
Leucine	16.9	33.0	250	1.12
Methionine	12.5	99.0	78.1	7.50
Phenylalanine	15.3	446	64.6	11.6
Tyrosine	10.3	257	15.0	7.05
Tryptophan	29.8	598	0	28.5
Aspartic Acid	41.0	1010	0	39.6

\* Transaminase activity is expressed as micromoles of L-glutamate formed per hour per mg. of enzyme preparation nitrogen; experimental details are given elsewhere (89).



no  $\alpha$ -ketoglutarate-isoleucine, valine activity, whereas  $\alpha$ -ketoglutarate-tryptophan, aspartate activity was limited to this fraction. On the other hand, fraction B catalyzed  $\alpha$ -ketoglutarate-isoleucine, valine transamination, but not reactions with tryptophan and aspartate.

An investigation was also made of the transaminase activities of a mutant of *E. coli*, which exhibited an absolute growth requirement for L-isoleucine, and a relative growth requirement for valine. The  $\alpha$ -keto analogues of isoleucine and valine did not support the growth of this mutant. Extracts of the mutant organism did not catalyze reactions between  $\alpha$ -ketoglutarate and valine or isoleucine, a finding compatible with the observed absolute requirement for isoleucine and inability of its keto analogue to support growth. The ability of the mutant to grow on media deficient in valine may be ascribed to the existence of an enzyme separately isolated (fraction C), which catalyzed the amination of  $\alpha$ -ketoisovalerate by transamination with alanine or  $\alpha$ -aminobutyrate (but not with other amino acids). The growth behavior of the mutant may therefore be explained in terms of a single block of the valine, isoleucine-glutamate reaction, and the relative requirement for valine by the persistence of the weaker valine-alanine,  $\alpha$ -aminobutyrate transaminase in the mutant. It is of interest that alanine or  $\alpha$ -aminobutyrate can substitute for valine in supporting the growth of the mutant. The fractionation studies and genetic considerations indicate that at least three separate transaminases exist in *E. coli*. Either there is some overlapping between the specificities of the enzymes of fractions A and B, or one or both of these fractions may represent more than one transaminase. Certain of these findings have been confirmed by Adelberg and Umbarger (115), namely, inability of the mutant to catalyze the glutamate-isoleucine valine reaction, and the existence of the valine-alanine system. These workers also found that the valine-alanine activity of the mutant was increased by repeated subculture in the absence of valine.

Studies on the glutamine and asparagine transaminase reactions indicated that these reactions were catalyzed by different enzymes, which were not identical with the classical glutamate-aspartate and



glutamate-alanine systems. Glutamate-phenylalanine and glutamate-tyrosine reactions are catalyzed by enzymes associated with the insoluble particles of rat liver (116), whereas certain glutamate-keto acid and the glutamine-keto acid reactions are catalyzed by enzymes of the soluble fraction.

Evidence for the existence of enzymes which catalyze leucine-alanine (103) and certain aliphatic amino acid-alanine (101) reactions has been reported. In each case the fractionation studies which have been carried out support the concept that these reactions are catalyzed by separate enzymes.

With the rapidly widening scope of transamination, it is not particularly remarkable that many new transaminase enzymes should come to light. It is of interest that those enzymes which have been thus far recognized appear to exhibit fairly specific substrate requirements. Although it might appear that virtually any amino acid might transaminate with any other in an intact tissue, the intermediate stages in such a transformation might well require catalysis by several separate enzymes.

#### *The Function of Vitamin B<sub>6</sub> in Transamination.*

The natural occurrence of pyridoxal and pyridoxamine, and the demonstration of their interconversion by nonenzymatic transamination reactions, led to the suggestion by Snell (117) that this vitamin plays a role in enzymatic transamination. Experimental evidence in support of this concept arose from studies on the effect of vitamin B<sub>6</sub> deficiency on transamination (118-120), and from investigations of purified enzymes which were activated by pyridoxal phosphate. Pyridoxal phosphate and pyridoxamine phosphate were found to activate the lowered liver transaminase activity of vitamin B<sub>6</sub>-deficient rats (119), and similar results were reported for the glutamate-aspartate transaminase of *S. faecalis* (121). These observations were consistent with the concept that interconversion between the aldehyde and amine forms of vitamin B<sub>6</sub> was involved in the mechanism of the reaction. However, it was found that pyridoxal phosphate, but not pyridoxamine phosphate, was capable of reactivating the purified pig heart glutamate-aspartate apotransaminase



(122). The latter finding was not compatible with the original hypothesis, and suggested the possibility of another type of mechanism. As yet, further support for an alternative hypothesis has not appeared.

Recent studies on the chemical structure and preparation of pyridoxal phosphate and pyridoxamine phosphate have facilitated further research in this area (123-128). Using crystalline pyridoxal and pyridoxamine phosphates (127-128), Meister, Sober, and Peterson (129) found that pyridoxal and pyridoxamine phosphates produced equivalent activation of the purified pig heart glutamate-aspartate apotransaminase. Maximal activation required preincubation of the enzyme with the coenzymes prior to addition of substrates. A somewhat longer preincubation period was required with pyridoxamine phosphate than with pyridoxal phosphate, a finding which may explain earlier results in which pyridoxamine phosphate was found to be relatively inactive. The requirement for preincubation may be interpreted as representing the time necessary for enzyme-coenzyme combination. After such combination has taken place, removal of the coenzyme is difficult, a finding suggesting relatively strong enzyme-coenzyme binding, and one which is consistent with the observed difficulty in resolving the transaminase originally. Conversion of pyridoxamine phosphate to pyridoxal phosphate prior to enzyme-coenzyme combination could not be demonstrated by enzymatic assay or by ultraviolet absorption studies. In view of the relationship between initial coenzyme concentration and activity (Fig. 1), it is improbable that the activating effect of pyridoxamine phosphate is due to even partial conversion to pyridoxal phosphate prior to enzyme-coenzyme combination. The evidence is compatible with the concept that both pyridoxamine phosphate and pyridoxal phosphate are coenzymes, and that interconversion between these forms of vitamin B<sub>6</sub> takes place in the presence of substrates after coenzyme-enzyme combination has occurred.

It has been demonstrated in a number of studies that deoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) is a very active vitamin-B<sub>6</sub> antagonist (130). In studies on tyrosine decarboxylase, Umbreit and Waddell (131) concluded that the effect of

this inhibitor was due to its conversion to deoxypyridoxine phosphate, which interfered with pyridoxal phosphate–decarboxylase combination. Experiments with the purified glutamate–aspartate apotransaminase revealed that deoxypyridoxine phosphate inhibited activation by pyridoxal or pyridoxamine phosphates by competing with the coenzymes for the enzyme (129). Pyridoxine phosphate did not acti-

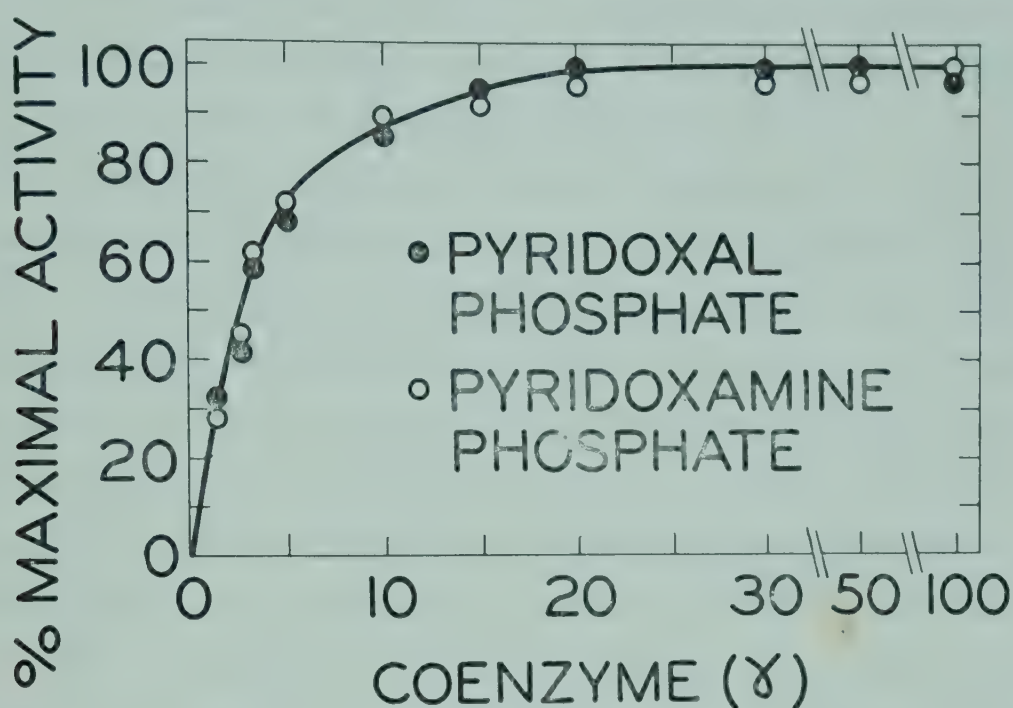
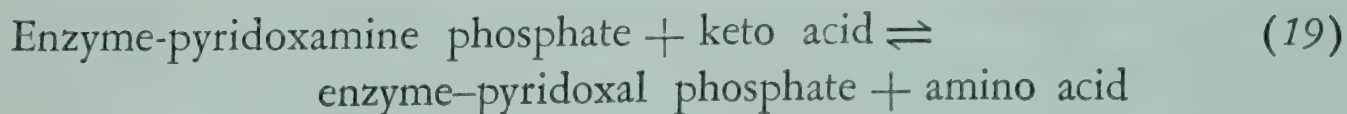
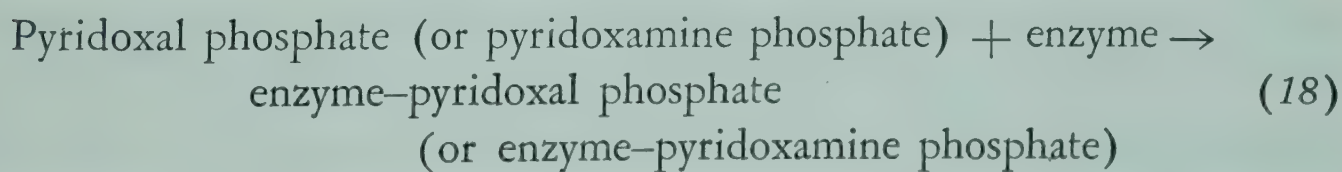


FIG. 1. Effect of coenzyme concentration on the activation of pig heart glutamate–aspartate apotransaminase (129).

vate the apotransaminase, but exerted a competitive inhibition of the type observed with deoxypyridoxine phosphate. The evidence suggests that deoxypyridoxine and pyridoxine phosphates are also tightly bound by the enzyme, although the affinity for the inhibitors is less than that for the coenzymes.

The available evidence suggests the following formulation:



Attempts to demonstrate conversion of enzyme–pyridoxamine phosphate to enzyme–pyridoxal phosphate, or reversal of this reaction, have not yet been successful. Studies with  $P^{32}$ -labelled



coenzymes indicated that approximately 0.1% of the added pyridoxamine phosphate was retained by the apotransaminase after prolonged dialysis, while with  $P^{32}$ -pyridoxal phosphate considerably more radioactivity remained associated with the enzyme after dialysis (129). These results indicate that an extremely small fraction of the added coenzyme is bound by the enzyme. Thus, in a typical experiment with 5 $\gamma$  of added pyridoxamine phosphate (cf. Fig. 1), not more than about 0.005 $\gamma$  combine with the enzyme. On the other hand, although 5 $\gamma$  of pyridoxal phosphate activate the apotransaminase to the same extent, about 0.06 $\gamma$  is bound by the enzyme preparation, a finding suggesting that pyridoxal phosphate combines with the enzyme at sites other than those required for activity.

A unique relationship between transamination and decarboxylation was observed with preparations of the aspartic acid- $\beta$ -decarboxylase of *C. welchii* (48). This enzyme requires catalytic amounts of keto acids or pyridoxal phosphate for activation, and the evidence suggests that the activity of the keto acid may be explained by a transamination reaction with pyridoxamine phosphate to yield pyridoxal phosphate. Thus, after ultraviolet irradiation, cell-free preparations could be activated by pyridoxal phosphate, but not by  $\alpha$ -keto acids. Added pyridoxamine phosphate did not activate except in the presence of an  $\alpha$ -keto acid. The pyridoxamine phosphate present in such preparations is non-dialyzable and appears to be bound to protein. It is therefore possible that the decarboxylase exists in combination with pyridoxamine phosphate, and is converted to the active (pyridoxal phosphate) form by transamination with  $\alpha$ -keto acids or with pyridoxal phosphate itself. The experimental difficulties involved in the demonstration of such a reaction appear similar to those which must be solved in attempting to demonstrate the proposed reversible transamination reaction (19). Such difficulties are due in part to the fact that only minute amounts of coenzyme are bound by the enzyme, and to the consequent problems involved in liberating and determining the coenzymes. A further complication is the occurrence of other types of coenzyme binding.

Most of the transaminases studied have been found to be activated by pyridoxal phosphate, and evidence for the presence of a coenzyme



in purified enzyme preparations has been reported (78, 83, 132). In addition to the classical glutamate-aspartate system, pyridoxal phosphate and pyridoxamine phosphate were found to activate the glutamate-valine, isoleucine transaminase fraction of *E. coli* (89). The glutamate-phenylalanine and glutamate-tyrosine reactions of *E. coli* (85), and the  $\alpha$ -ketoglutarate-leucine, isoleucine, tyrosine, tryptophan, methionine reactions of heart muscle are activated by pyridoxal phosphate (86). In contrast to the glutamate-alanine and cysteine desulfhydrase activities, the glutamine transaminase activity of the livers of vitamin B<sub>6</sub>-deficient rats showed no decrease in activity (133). Although there is thus far no evidence for the function of vitamin B<sub>6</sub> in the glutamine transaminase reaction, its participation cannot be excluded. There is probably great variation among the vitamin B<sub>6</sub> enzymes in the stability of enzyme-coenzyme binding. Thus, cysteine desulfhydrase of rat liver may be readily resolved by dialysis, whereas this procedure does not affect the glutamate-alanine transaminase (133).

The effectiveness of the  $\alpha$ -keto analogues of certain amino acids in replacing the corresponding amino acids in supporting the growth of animals and microorganisms has been demonstrated in a number of studies. Holden, Wildman, and Snell (134) have shown that several microorganisms could be grown on media deficient in vitamin B<sub>6</sub>, provided all of the essential amino acids were present. On the other hand, when the analogous  $\alpha$ -keto acids were supplied in place of the amino acids, little or no growth occurred. The  $\alpha$ -keto acids were utilized for growth only when the media were supplemented with high levels of vitamin B<sub>6</sub>. The requirement for vitamin B<sub>6</sub> is compatible with the concept that the  $\alpha$ -keto acids are converted to the corresponding amino acids by transamination.

A number of studies have been carried out on model nonenzymatic transaminase reactions involving a metal ion, pyridoxal, pyridoxamine, and various amino and keto acids. Recently Metzler, Ikawa, and Snell (135) have proposed a general mechanism for vitamin B<sub>6</sub>-catalyzed reactions, which represents an elegant extension and elaboration of the concept of Schiff's base formation previously proposed. The analogy between the enzymatic and nonenzymatic systems is



close, although as yet participation of metal ions in enzymatic transamination has not been shown. It has been suggested that the enzyme itself may function in place of the metal ion. Experiments on highly purified transaminases may be expected to shed light on this problem.

Results bearing on the mechanism of transamination have been obtained in studies on the labilization of the  $\alpha$ -hydrogen atom of amino acids (78, 136, 137). Thus, when  $\alpha$ -deuteroalanine and glutamate were incubated with transaminase preparations, almost all of the deuterium was found in the aqueous medium. Exchange of the  $\alpha$ -hydrogen atom also occurs in the absence of  $\alpha$ -keto acids, but not with N-methylamino acids. Other aspects of this approach, including the ability of boiled transaminase to catalyze labilization of the  $\alpha$ -deuterium atom, deserve further attention.

Appreciable nonenzymatic transamination has been observed between glyoxylate and glutamine (and other amino acids), without added metal ion, at physiological values of pH and room temperature (138). It is possible that glyoxylate, representing a special case, may enzymatically transaminate directly with amino acids, and thus not require the participation of a vitamin B<sub>6</sub> derivative.

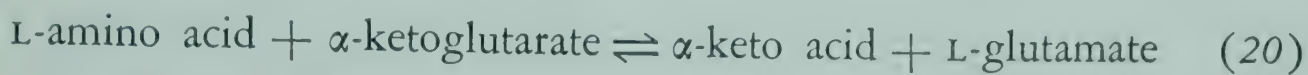
Although the function of pyridoxal phosphate as a coenzyme was recognized first in decarboxylase and transaminase reactions, it is now recognized that pyridoxal phosphate is also the coenzyme for a number of other enzymes which catalyze reactions involving amino acids, e. g., cysteine desulfhydrase (139), amino acid racemases (36), kynureninase (140), tryptophanase (141), the tryptophan-synthesizing system (142), and serine dehydrase (31). It is evident that vitamin B<sub>6</sub> plays an important role, not only in transamination and decarboxylation, but also in certain specific reactions of individual amino acids.

### *The Role of Transamination in Metabolism.*

The widespread occurrence of transamination in animals, plants, and microorganisms suggests that this reaction may be of general significance in nitrogen metabolism. It has long been recognized that transamination represents a mechanism for interconversion



between glutamate, aspartate, and alanine, and intermediates of the Krebs cycle. Furthermore, it is probable that transamination provides a mechanism for the oxidation of many L-amino acids as described by the following reactions:



These reactions occur at a rate sufficiently rapid to account for the oxidation of L-amino acids in animal tissues observed by Krebs. According to this concept, the glutamic dehydrogenase system represents an important link between the metabolism of amino acids and carbohydrates, and a significant pathway in the conversion of amino acid nitrogen to other nitrogen-containing products. The oxidation of certain amino acids may be catalyzed to some extent by direct oxidative deamination, although this would appear to be a relatively minor pathway in the tissues of higher animals.

The initial step in the degradative metabolism of many amino acids involves removal of the  $\alpha$ -amino group, and it appears likely that this reaction is accomplished by transamination. Thus, workers in three laboratories have agreed that transamination is the initial step in the metabolism of tyrosine (143-145). The evidence which has accumulated on the metabolic degradation of leucine (146), isoleucine (147), and valine (148) indicates an initial loss of the  $\alpha$ -amino group, which probably occurs by transamination. Investigations of cysteine metabolism suggest that transamination occurs at the stages of cysteine sulfinic and cysteic acids, leading to sulfinylpyruvate and sulfopyruvate, respectively (149). It is also possible that cysteine itself transaminates to  $\beta$ -mercaptopyruvic acid which is desulfurated to pyruvate (92).

Although earlier studies suggested that lysine did not participate in reversible transamination in the rat (80), there is now evidence that lysine is converted to pipecolic acid and to  $\alpha$ -aminoadipic acid by transformations which probably involve transamination (150). The pathway in *Neurospora* may be similar, although it is not identical with that observed in the rat. Transamination of the  $\alpha$ -amino group of lysine would yield  $\alpha$ -keto- $\epsilon$ -aminocaproic acid, which



appears to be in equilibrium with  $\Delta'$ -piperidine-2-carboxylic acid (97). The latter compound may undergo optically specific reduction to L-pipecolic acid. It is also possible that  $\alpha$ -aminoadipic- $\delta$ -semi-aldehyde (or an  $\alpha$ -N-substituted derivative, cf. ref. 105) is an intermediate in these transformations. The  $\omega$ -aldehyde could arise by an  $\omega$ -amino group transamination, or by hydrolysis of  $\Delta'$ -piperidine-6-carboxylic acid (150). Transamination of  $\omega$ -N-substituted lysine and analogous  $\alpha$ -keto acids has been demonstrated (88, 99); the possible role of such reactions remains to be elucidated.

The rapid transamination of glyoxylate with glutamine and certain other amino acids in systems in vitro is of interest in view of the findings of Weinhouse and Friedmann (150a), who observed conversion of glyoxylate to glycine in the rat.

The significance of transamination in the metabolism of methionine, histidine, and phenylalanine is not yet clear. Although these amino acids have been observed to transaminate rapidly in several tissues, their metabolism would appear to proceed predominantly along other pathways. Similarly, although tryptophan is capable of participating in transamination, the significance of this reaction in the total picture of tryptophan metabolism remains to be assessed. On the other hand, transamination of kynurenine appears to be of importance in the formation of kynurenic acid. Miller et al. (151) have recently found that cells of a strain of *Pseudomonas* catalyze transamination between kynurenine and  $\alpha$ -ketoglutarate to form glutamic and kynurenic acids. The intermediate formation of *o*-aminobenzoylpyruvate is assumed, although this compound was not detected. Recently, it has been concluded that transamination plays a role in the metabolism of diiodotyrosine in liver and kidney (152).

It has also become apparent that transamination represents an essential step in the biosynthesis of certain amino acids. Thus, it is probable that the final step in the biosynthesis of valine, isoleucine (89), and phenylalanine (153, 154) in *E. coli* is transamination involving the analogous  $\alpha$ -keto acids. As stated above, transamination is involved in ornithine biosynthesis in *E. coli* (105), and a transamination step may also occur in the biosynthesis of histidine (155).



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# SOME ASPECTS OF AMINO ACID METABOLISM IN BRUCELLAE AND ITS RELATIONSHIP TO PANTOTHENIC ACID

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STUDIES BY Goodlow, Braun, and coworkers (1) on factors influencing the establishment of nonsmooth mutants in originally smooth cultures of *Brucella* in liquid synthetic medium have shown that these population changes are correlated in some way with the formation of an amino acid, apparently  $\alpha$ -alanine, and with the pantothenic acid supply. Earlier investigations by us have revealed some of the pathways of carbohydrate metabolism (2) and have established that  $\alpha$ -alanine may be synthesized by several transamination reactions and perhaps by a direct amination of pyruvate (3, 4).

## STEREOSPECIFIC ASPARAGINASES

Since asparagine is the sole amino acid present in the synthetic medium employed and since the isomeric configuration of this compound bears a direct relationship to the amount and type of population change that will occur, some aspects of asparagine metabolism of whole resting cells and of sonic extracts have been investigated using the smooth strain 19 of *Brucella abortus*. Chromatographs of filtrates of aged cultures demonstrated the presence of free aspartic acid, presumptive evidence for asparaginase activity. Whole resting cells were able to hydrolyze either D-or L-asparagine at nearly comparable rates. The same result has been obtained with freshly prepared, dialyzed sonic extract; however, usually D-asparagine hydrolysis is somewhat more rapid. Repeated freezing and thawing of the sonic extract rapidly inactivated L-asparaginase, whereas D-asparaginase was only moderately affected by this treatment. If the freezing and thawing was carried out a sufficient number of times,



only D-asparaginase remained and L-asparaginase was not detectable. Attempts at reactivation of L-asparaginase in such preparations by metallic cations, cysteine or thioglycollate, or pyridoxal phosphate were unsuccessful. The addition of metallic cations, versene, or 2, 2'-dipyridyl to freshly prepared, dialyzed extract did not alter the rate of hydrolysis of either asparagine isomer, and it appears that readily dissociable metal ion cofactors are not involved.

Since the configuration of the resulting aspartic acid was unknown, it was considered possible that there was only one asparaginase plus a racemase that converted the inactive form to the antipode, which could be hydrolyzed. Pyridoxal phosphate addition, however, did not alter the hydrolysis rate of either isomer by fresh extracts and, as stated before, was unable to activate L-asparaginase following freezing-thawing inactivation. It was shown that upon hydrolysis L-asparagine yields L-aspartic acid and D-asparagine yields D-aspartic acid. The extracts contain many transaminases, among the most active being aspartic-glutamic transaminase. By adding  $\alpha$ -ketoglutarate to extracts hydrolyzing the isomers of asparagine, it was demonstrated that L-asparagine gave rise to aspartate and glutamate and that D-asparagine gave rise to aspartate and a small amount of glutamate that could be accounted for by direct amination of  $\alpha$ -ketoglutarate. There was no evidence of racemization of either asparagine or aspartate.

A study of the relationship of pH to the rate of hydrolysis revealed that D-asparaginase possessed a rather broad pH-activity range, with a maximum around 8.0, while L-asparaginase was only weakly active below pH 7.0 and increased sharply at pH 8.0.

Both enzymes were strongly inhibited by *p*-chloromercuribenzoate and by N-ethylmaleimide, but iodoacetate or Furacin was ineffective. There was some reversal of the inhibition produced by these substances by means of cysteine, and it has been concluded that both enzymes possess free sulfhydryl groups essential for activity.

Attempts at separation of the asparaginases by fractional ammonium sulfate precipitation failed. Acetic acid precipitation in the cold, however, effected a partial separation of these enzymes. Pre-

precipitates obtained from the sonic extract at pH values of 5.0, 4.5, and 4.0 were dissolved in pH 8.0 buffer and repeatedly precipitated at the same pH values. L-asparaginase precipitated more sharply than D-asparaginase, and the 4.5 precipitate was relatively rich in

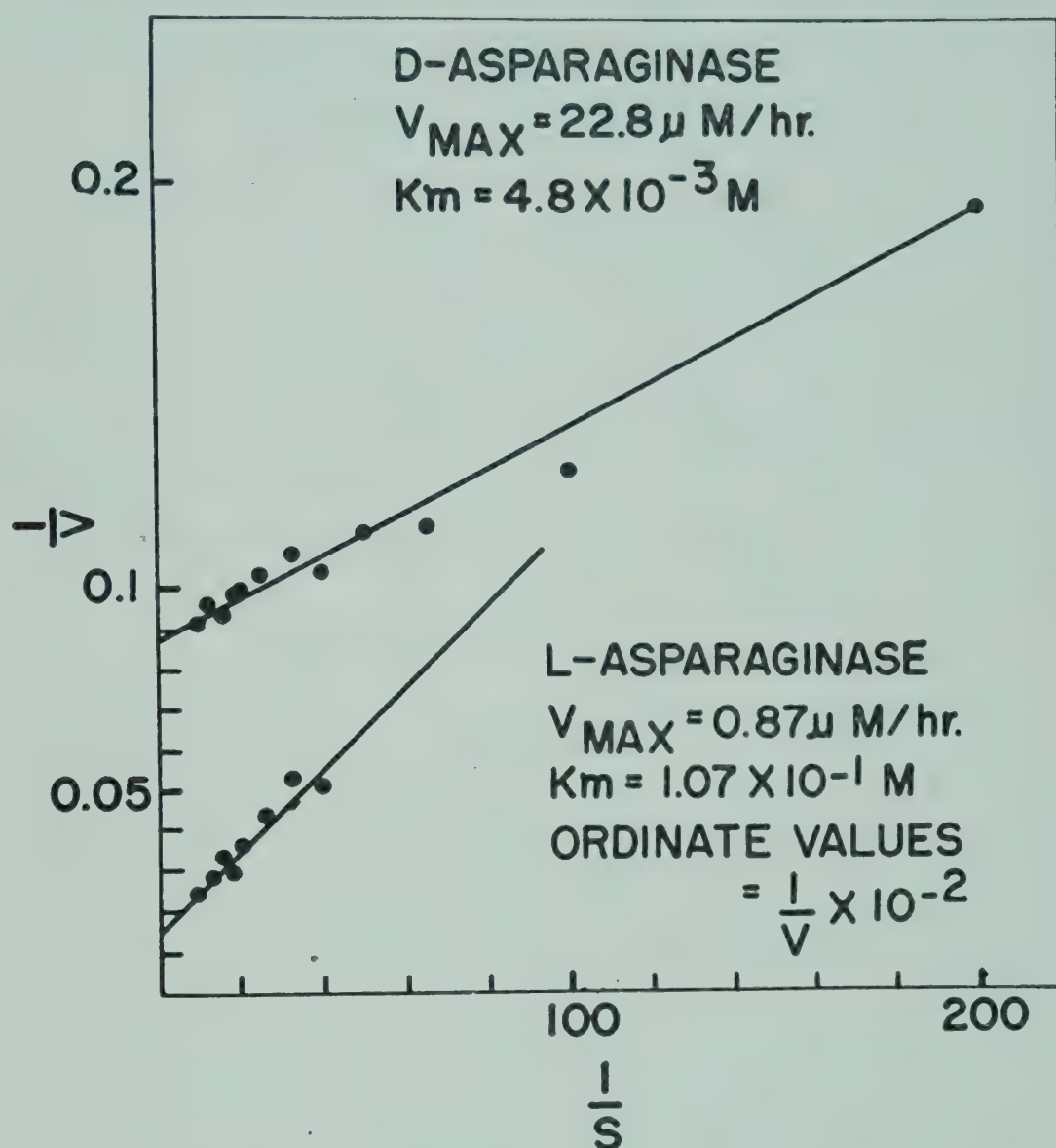


FIG. 1. Reaction velocity versus substrate concentration for L-asparaginase and D-asparaginase.

One ml. of sonic extract plus 1 ml. of substrate solution incubated anaerobically in Thunberg tubes at 37° C. Final volume, 2 ml., pH 8.0. Velocity expressed as micromoles aspartic acid liberated per hour. Substrate concentration expressed as final molarity.

L-asparaginase. Repetition of the fractionation procedure yielded, after several additional steps, L-asparaginase free of D-asparaginase, but the overall losses are very great.

A survey of the velocity of hydrolysis as a function of substrate concentration offered additional evidence of the dissimilarity of the stereospecific asparaginases. Fig. 1 shows a double reciprocal plot



of these data, both enzymes giving a linear relationship but yielding greatly differing Michaelis constants. These data have only recently been published (5).

Racemic asparagine is hydrolyzed by whole cells and by sonic extracts at a rate nearly equivalent to an equimolar quantity of either isomer alone, and there appears to be no competitive inhibition by either isomer with the deamidation of the other. A direct test of this was performed with an extract devoid of L-asparaginase. It was found that L-asparagine did not interfere with hydrolysis of D-asparagine; and presumably the reverse situation is true. In Warburg experiments with resting cells it has been possible to show definite D-asparagine interference with the oxidation of L-asparagine (6). This interference must be exerted at some site other than that of deamidation or transamination and probably concerns an oxidative degradation of L-aspartate.

#### PANTOTHENATE SYNTHESIS BY RESTING CELLS

Population studies by Goodlow and coworkers (7) have demonstrated that the configuration of asparagine in the liquid synthetic medium has a profound influence on the nature and extent of population changes in originally smooth cultures. L-asparagine medium maintains the smooth type, and few if any nonsmooth mutants establish themselves, whereas DL-asparagine medium promotes a comparatively rapid establishment of nonsmooth types and D-asparagine medium gives rise to a very high percentage of nonsmooth types, although growth is sparse. Further investigations by Mika, Braun, and coworkers (8) revealed that an increase in pantothenic acid concentration above the usual 40 m $\mu$ g./ml. suppressed such population changes. Further studies in our laboratory (9) showed that in L-asparagine medium, pantothenic acid is synthesized by smooth organisms, and the concentration of pantothenate rises to over 100 m $\mu$ g./ml. after sufficient incubation time. However, addition of an equimolar amount of D-asparagine to the medium at any time prevents the further synthesis and accumulation of pantothenate and simultaneously favors the establishment of nonsmooth types. In

contrast, nonsmooth types (rough) growing in L-asparagine medium take up pantothenate from the medium and reduce the concentration to about 15 m $\mu$ g./ml. after an identical incubation period. In view of these relationships between population change and metabolism, the synthesis of pantothenate by whole smooth cells and sonic extracts from various amino acids was studied. It was found that

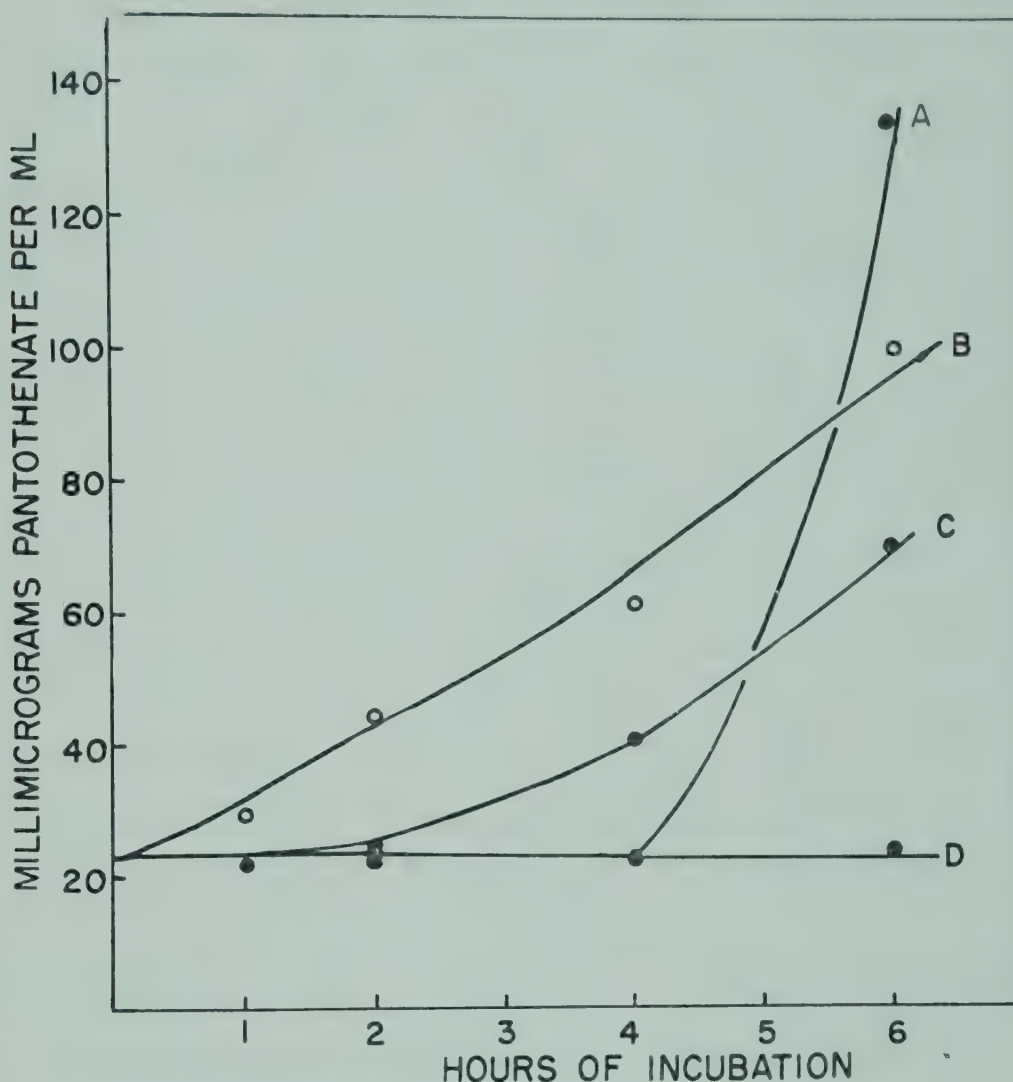


FIG 2. Synthesis of pantothenic acid from L-valine by smooth *Brucella abortus*, strain 19.

5 ml. of cell suspension plus substrates diluted to 20 ml. with 0.1 M phosphate buffer, pH 7.4. Incubated on shaker at 37° C. and sampled at times indicated. Complete system contained .01 M L-valine, .01 M  $\beta$ -alanine, and 0.1 M glucose as final concentration. Curve A, minus glucose; Curve B, complete system; Curve C, minus  $\beta$ -alanine; Curve D, minus valine. Base line of 23 m $\mu$ g. pantothenate per ml. present in cell suspension in complete absence of substrate.

whole cells synthesize pantothenate readily from pantoil lactone or potassium pantoate plus  $\beta$ -alanine. None is produced in the absence of pantoate or its precursor. Either L-valine or D-valine serves as an excellent source of pantoate for the synthesis of pantothenate.



Ketovaline is also readily utilized; however, the cells are unable to mobilize endogenous sources of pantoate, valine, or their precursors.  $\beta$ -alanine is present endogenously in nearly maximal amounts (Fig. 2). In all these studies glucose was routinely added as an energy source.

Next, it was established that the synthesis of pantothenic acid from L-valine plus  $\beta$ -alanine is markedly inhibited by either L-leucine or L-isoleucine. L-leucine is more inhibitory on a molar basis, as anticipated because of its close structural similarity to valine. In this connection it is of interest that cultures of smooth cells in synthetic medium containing only leucine as the nitrogen source exhibit rather rapid population changes and establishment of nonsmooth types.

The large endogenous supply of  $\beta$ -alanine or its precursors precluded investigation of  $\beta$ -alanine precursors under these conditions, notably at pH 7.4. It was found feasible to reduce the endogenous  $\beta$ -alanine greatly by reduction of pH to 5.4, and it was then possible to demonstrate  $\beta$ -alanine formation from both L-asparagine and L-aspartic acid. A number of variables were involved in this phenomenon, namely, age of cells, substrate concentration, and pH, which are all controlling factors in the production of  $\beta$ -alanine from either L-asparagine or L-aspartate. Under some conditions these substrates may also markedly inhibit pantothenate synthesis. These results may help to explain certain anomalies previously observed, namely, that aspartate inhibited pantothenate synthesis although stimulation might be expected by formation of  $\beta$ -alanine. Employing this system at pH 5.4, it could be shown that D-asparagine in equivalent amount inhibited by 60-65% the conversion of L-asparagine to  $\beta$ -alanine. Neither D- nor L- $\alpha$ -alanine affected pantothenate synthesis under a variety of conditions.

At pH 7.4, none of the logical one-carbon sources succeeded in accelerating the rate of pantothenate synthesis from L-valine plus  $\beta$ -alanine. At pH 5.4, however, L-serine accelerated this rate in proportion to the concentration added, and this acceleration is competitively inhibited by D-serine. This reaction was not readily detectable at pH 7.4, although there is some evidence that prolonged



starvation of resting cells reduces the endogenous one-carbon sources to a level low enough to be enhanced by the addition of L-serine.

#### PANTOTHENATE SYNTHESIS BY CELL-FREE EXTRACTS

Studies with dialyzed sonic extract have demonstrated the presence of an active coupling enzyme which combines pantoate and  $\beta$ -alanine to produce pantothenate. This reaction is ATP-dependent and is stimulated by small amounts of magnesium ion. The Michaelis constant for K pantoate is  $5.1 \times 10^{-2} M$  while the  $K_m$  for  $\beta$ -alanine is  $4 \times 10^{-5} M$ , an unexpectedly low figure. Such a high affinity of the enzyme for  $\beta$ -alanine readily explains the difficulty encountered in reducing the endogenous  $\beta$ -alanine supply in whole cells. Efforts to synthesize pantoate from valine in sonic extract initially met with failure even though the initial reaction, namely, transamination to  $\alpha$ -ketoglutarate producing ketovaline, occurred readily. Endogenous pantothenate rapidly disappears from these extracts upon the addition of ATP or in the absence of valine. Unexpectedly,  $\alpha$ -ketoglutarate was found to accelerate pantothenate disappearance. The characteristics of this system are being investigated at present. Results of experiments designed to show  $\beta$ -alanine synthesis from L-asparagine or L-aspartic acid have been inconclusive, although L-asparagine apparently does yield  $\beta$ -alanine at a slow rate.

To summarize, cells of smooth *B. abortus*, strain 19, contain stereospecific asparaginases. Neither isomer of asparagine interferes with the deamidation of the other; however, interference with L-asparagine oxidation by D-asparagine can be readily demonstrated. With suitable conditions L-asparagine yields  $\beta$ -alanine, which is utilized for pantothenic acid synthesis; and D-asparagine interferes with this conversion. These observations may be correlated with studies on population changes where D-asparagine has been found to promote the establishment of nonsmooth types in originally smooth cultures and where surplus pantothenic acid antagonized this effect. Whole cells can synthesize pantothenic acid from L- or D-valine plus  $\beta$ -alanine, and this synthesis is markedly stimulated by L-serine. D-Serine competitively inhibits such stimulation by L-serine. Synthesis



of pantothenate from L-valine plus  $\beta$ -alanine is antagonized by L-leucine or L-isoleucine. These data, together with the results of studies on population changes, suggest that pantothenic acid synthesis and utilization are critical areas in the metabolism of *Brucella* and that disturbance of the normal processes by addition of inhibitory amino acids or by insufficient synthesis of other amino acids might easily provide selective conditions leading to population changes involving the replacement of one type by a fitter mutant type.

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# TRANSAMINATION OF D-AMINO ACIDS

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IN STUDIES on the mechanisms of synthesis of D-glutamic acid and D-glutamyl polypeptide by *Bacillus subtilis*, it was observed that cell-free extracts catalyze transamination reactions involving D-amino acids (6). Some of the results of experiments on the transamination of D-amino acids are summarized here.

## METHODS

Cell-free extracts of *B. subtilis*, ATCC 9945, were prepared by treating a thick suspension of cells in a Raytheon sonic oscillator. The sonic-treated material was centrifuged, and the clear supernatant solution was used in the enzyme studies.

For testing transaminase activity, reaction mixtures were usually made up to contain 0.1 ml. of enzyme, 20  $\mu$ g. of pyridoxal phosphate, 100  $\mu$ M of the appropriate substrates, and 50  $\mu$ M of phosphate at pH 8.0, in a final volume of 1 ml. The mixtures were incubated in stoppered tubes at 37° C. for the desired period of time, and the reaction was stopped by holding the tubes in boiling water for 5 minutes. No reaction was detected when boiled enzyme was tested.

Total glutamic acid, aspartic acid, and alanine were determined quantitatively by paper chromatography (2). L-Glutamic acid was determined manometrically with decarboxylase from *Escherichia coli* (8). D-Glutamic acid was estimated by subtracting the amount of the L-isomer from the total. D-Alanine was determined manometrically with D-amino acid oxidase from hog kidneys (4). Pyruvic and  $\alpha$ -ketoglutaric acids were determined by a modification of the method of Cavallini et al. (1), in which the 2, 4-dinitrophenyl hydrazones are separated by paper chromatography. Phenol-water (3:1) was used as the solvent. Nitrogen was determined by the method of Johnson (3).



## RESULTS

*Transaminase Activities of Sonic Extracts.*

Glutamic acid was produced when sonic extracts were incubated with  $\alpha$ -ketoglutaric acid and either isomer of aspartic acid or alanine. The glutamic acid formed when L-aspartic acid was the added amino donor was largely the L-isomer, and that formed when D-aspartic acid was the added amino donor was largely the D-isomer. When crude extracts were incubated with  $\alpha$ -ketoglutaric acid alone, a small amount of glutamic acid was produced, and it was a mixture of the D- and L-isomers. This could be reduced, but not eliminated, by dialysis.

Extracts contained an active alanine racemase (5, 9) which converted both isomers of alanine to the racemic mixture. Most of the glutamic acid produced when either L- or D-alanine and  $\alpha$ -ketoglutaric acid were incubated with extracts was the D-isomer, indicating that D-alanine transaminated more rapidly than L-alanine.

When D-aspartic acid was incubated with  $\alpha$ -ketoglutaric acid, alanine was formed in addition to glutamic acid. This is believed to result from transamination with pyruvic acid which originated from the oxaloacetic acid produced from aspartic acid. It was observed that when oxaloacetic acid was incubated with D-aspartic acid and enzyme, alanine was formed, and when D-glutamic acid and oxaloacetic acid were incubated with enzyme, both aspartic acid and alanine were formed.

D-Aspartic acid, as well as D- and L-glutamic acid, transaminated with pyruvic acid to form alanine. The resulting alanine was a mixture of the two isomers, since alanine racemase was present in the extracts. No detectable amounts of alanine were formed from L-aspartic acid and pyruvic acid.

*Relative Stabilities of D- and L-Amino Acid Transaminases.*

The data in Table 1 compare the stabilities of the D- and L-amino acid transaminases. Fresh extracts were active with both D- and L-aspartic acid in the synthesis of glutamic acid from  $\alpha$ -ketoglutaric

TABLE 1  
RELATIVE STABILITIES OF L- AND D-AMINO ACID TRANSAMINASES

Prep. No.	Treatment	Glutamic acid produced from		
		$\alpha$ -Kg	$\alpha$ -Kg + L-Asp	$\alpha$ -Kg + D-Asp
		$\mu$ M/ml.	$\mu$ M/ml.	$\mu$ M/ml.
I	None (fresh extract)	2.2	24.8	26.5
II	Prep. I dialyzed 70 hours at 5° C. (0.01 M phosphate buffer, pH 7.4)	0.8	2.1	11.0
III	Prep. I stored 72 hours at 5° C.	3.1	5.4	27.5

\* 0.1 ml. of enzyme, 20  $\mu$ g. of pyridoxal phosphate, and 100  $\mu$ M of appropriate substrates in a final volume of 1.0 ml.; 0.01 M phosphate buffer, pH 7.4. Incubated 2 hours. Abbreviations in table:  $\alpha$ -Kg,  $\alpha$ -ketoglutaric acid; Asp, aspartic acid.

acid, but the activity with D-aspartic acid was usually much greater than that with the L-isomer. When extracts were allowed to stand at 5° C. for 72 hours, the activity with D-aspartic acid was maintained, but that with L-aspartic acid was greatly reduced. When extracts were dialyzed for 70 hours, the activity with both isomers of aspartic acid was reduced, but that with the L-isomer was reduced much more than that with the D-isomer. These results were obtained with pyridoxal phosphate added to the reaction mixtures, and thus the loss in activity upon dialysis could not be restored completely by the addition of coenzyme.

### *Effect of pH.*

The optimum pH for the reaction between  $\alpha$ -ketoglutaric acid and D-aspartic acid was in the range of 8.5 to 9.0. The same reaction with L-aspartic acid proceeded at an optimum rate over a much broader pH range of 7 to 8.

### *Effect of Pyridoxal Phosphate.*

The effect of pyridoxal phosphate on the transamination reaction between D-aspartic acid and  $\alpha$ -ketoglutaric acid was studied. A preparation was used that had been partially purified by ammonium



sulfate fractionation. The results are shown in Fig. 1. Under the conditions of the test 20 to 40  $\mu$ g. of added pyridoxal phosphate per ml. were required for maximum activity.

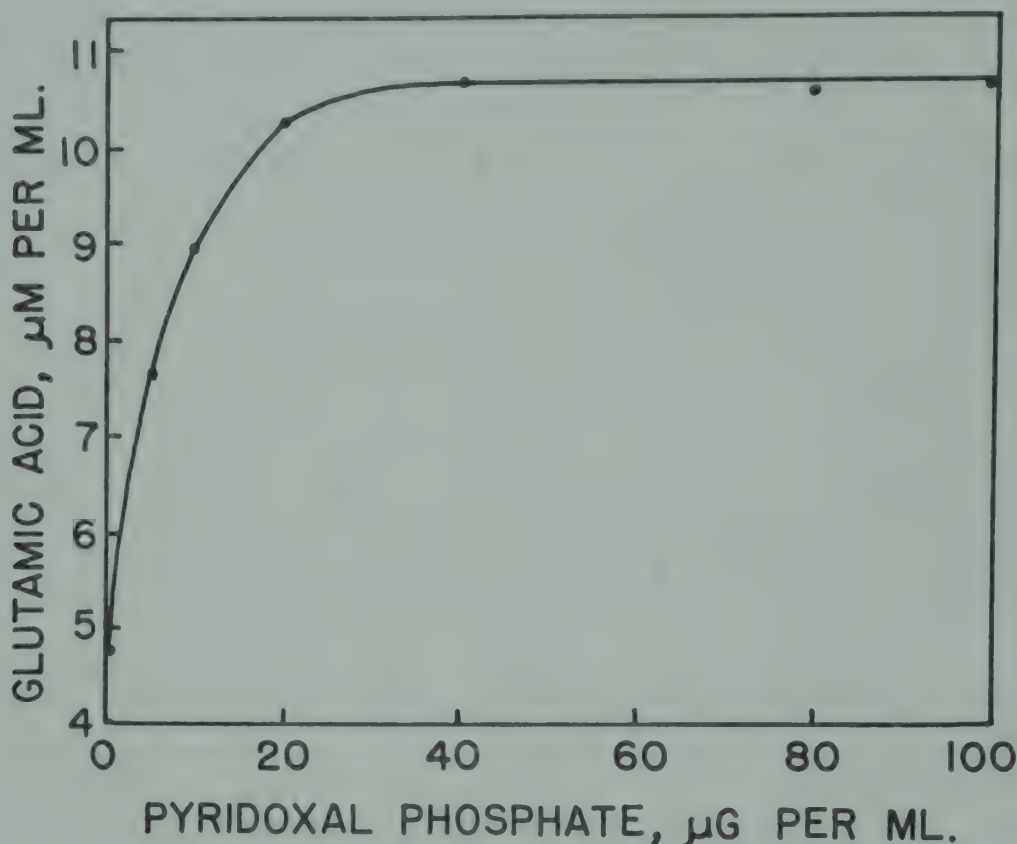


FIG. 1. Effect of pyridoxal phosphate on the synthesis of glutamic acid from  $\alpha$ -ketoglutaric acid and D-aspartic acid. Reaction mixtures were made up to contain 0.1 ml. of enzyme (0.15 mg. of nitrogen), 100  $\mu$ M of  $\alpha$ -ketoglutaric and aspartic acids, and pyridoxal phosphate as shown, in 1.0 ml. of 0.05 M phosphate buffer at pH 8.0. Tubes were incubated 35 minutes.

#### *D-Amino Acid Transaminase Activity of a Partially Purified Preparation.*

By fractionation with ammonium sulfate, it was possible to obtain a preparation which was active with D-amino acids but inactive with the L-isomers. With such a preparation both D-aspartic acid and D-glutamic acid were active in transaminating with pyruvic acid, but the L-isomers were inactive. With this same preparation D-aspartic acid transaminated rapidly with  $\alpha$ -ketoglutaric acid, and the resulting glutamic acid was entirely the D-isomer. L-Aspartic acid was inactive. Glutamic acid was produced when  $\alpha$ -ketoglutaric acid and either D- or L-alanine were incubated together with the enzyme, but the D-isomer was the more active of the two. The apparent activity with L-alanine is probably a result of the presence of alanine racemase

in the partially purified transaminase preparation. This was not tested. With the purified preparation no amino acids were produced from  $\alpha$ -ketoglutaric acid or pyruvic acid in the absence of added amino donors.

With 0.1 M  $\alpha$ -ketoglutaric acid and D-aspartic acid and an incubation period of 30 minutes, the purest preparation thus far obtained catalyzed the synthesis of 60  $\mu$ M of glutamic acid per mg. of enzyme nitrogen when a concentration of 0.15 mg. of enzyme nitrogen per ml. was used.

#### *Transamination Balance.*

Balance studies were done on the transamination reaction involving the synthesis of glutamic acid from  $\alpha$ -ketoglutaric acid and D-alanine. A preparation was used which had been partially purified by ammonium sulfate fractionation. Upon incubating 42.6  $\mu$ M of D-alanine and 39.8  $\mu$ M of  $\alpha$ -ketoglutaric acid with enzyme for 1 hour, 15.1  $\mu$ M of glutamic acid and 15.0  $\mu$ M of pyruvic acid were produced. During the same time 15.6  $\mu$ M of  $\alpha$ -ketoglutaric acid and 15.0  $\mu$ M of alanine were used. In the same experiment the reverse also was studied. From 44.3  $\mu$ M of D-glutamic acid and 25.7  $\mu$ M of pyruvic acid, 20.1  $\mu$ M of alanine and 20.6  $\mu$ M of  $\alpha$ -ketoglutaric acid were produced. During the same time 19.2  $\mu$ M of glutamic acid and 18.8  $\mu$ M of pyruvic acid were used. These data provide conclusive evidence that the reactions are true transamination reactions.

#### *Transamination of Other D-Amino Acids.*

Crude sonic extracts were active in the synthesis of glutamic acid from  $\alpha$ -ketoglutaric acid and the D- and L-isomers of methionine and serine. Partially purified enzyme preparations were active with the D-isomers only. However, the activity with these amino acids was not nearly as great as that with D-aspartic acid or D-alanine. None of a series of other D-amino acids including phenylalanine, leucine, isoleucine, valine, histidine, threonine, and tryptophan was significantly active in transamination with  $\alpha$ -ketoglutaric acid when a crude extract was tested.



## DISCUSSION

The optimum medium for production of glutamyl polypeptide by growing cultures of *B. subtilis* contains a large amount of L-glutamic acid. The glutamic acid of the polypeptide contains from 20 to 80 per cent of the D-isomer (7). Although a glutamic acid racemase has not been demonstrated in this organism, the finding of these D-amino acid transamination reactions suggests the following series of reactions as a mechanism for an indirect conversion of L-glutamic acid to the D-isomer: (1) transamination between pyruvic acid and L-glutamic acid produces L-alanine and  $\alpha$ -ketoglutaric acid; (2) the alanine is racemized; and (3) transamination between  $\alpha$ -ketoglutaric acid and D-alanine results in the formation of D-glutamic acid.

The discovery of D-amino acid transamination suggests an important role for alanine racemase. It appears that in *B. subtilis*, alanine may be the key compound involved in D-amino acid metabolism. It will be interesting to test other organisms and tissues for D-amino acid transamination and to learn whether the reactions occur in a number of species or whether they are peculiar to *B. subtilis* and closely related organisms.

## SUMMARY

Sonic extracts of *B. subtilis* catalyzed a series of transamination reactions involving D-amino acids. D-Glutamic acid was synthesized from  $\alpha$ -ketoglutaric acid and D-aspartic acid or D-alanine. Alanine was produced from pyruvic acid and D-aspartic acid or D-glutamic acid. Extracts contained an active alanine racemase which converted both isomers of alanine to the racemic mixture. When fresh extracts were used, L-amino acids were also active in transamination, but upon aging of extracts the activity with L-amino acids disappeared or was reduced, while the activity with D-amino acids was maintained. By fractionating with ammonium sulfate it was possible to obtain preparations which were specific for D-amino acids.

D-Methionine and D-serine were also active in the synthesis of glutamic acid from  $\alpha$ -ketoglutaric acid, but the activity with these amino acids was less than that with D-aspartic acid and D-alanine.

The pH optimum for the reaction between  $\alpha$ -ketoglutaric acid and D-aspartic acid was in the range of 8.5 to 9.0.

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# REACTIONS OF ALPHA-METHYL AMINO ACIDS

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IT OCCURRED to us that perhaps something could be learned about amino acid metabolism and the enzymes involved by the preparation and study of a variety of amino acids in which a very minor structural alteration had been accomplished. For this purpose we chose to alter the  $\alpha$ -hydrogen and replace it with a methyl group. This was accomplished by Doctors Leanza, Conbere, Matzuk, Rogers, and Pfister of the Research and Development Division of MERCK & Co., Inc. While a great many amino acid analogues have been prepared in the past, the structural change involved in substituting a methyl for the  $\alpha$ -hydrogen appeared more subtle than most, particularly since one of the naturally occurring amino acids, alanine, is actually the  $\alpha$ -methyl derivative of another, glycine.

However, we were somewhat surprised to find how few  $\alpha$ -methyl amino acids had been studied in the past.  $\alpha$ -Methylalanine and  $\alpha$ -methylserine were found to be without effect on a bacterial system (1), but these are the only studies on the biological effect of  $\alpha$ -methyl amino acids before 1952, although the preparation of several others is described in the chemical literature.

The first substance given serious study was  $\alpha$ -methylglutamic acid, with the results shown in Table 1. By inhibition we imply that the amount of the  $\alpha$ -methyl derivative is somewhat related to the amount of glutamic acid; that is, equal to or twice as much or five-fold. We regard inhibitions which may be observed at 100-fold or 1000-fold concentration as having little significance. Where inhibition occurs, the usual inhibition index is of the order of 0.5 to 5 rather than of the order of 100 to 1000. Where inhibition has been studied in detail, it proved to be uncompetitive. That is, if the  $\alpha$ -methylglutamate and the glutamate are added to the enzyme system simul-

TABLE 1  
GLUTAMIC ACID

	(DL) $\alpha$ -methyl	Ref.
Decarboxylation	inhibits	(14, 17)
Dehydrogenation	inert	(12, 17)
Transamination	inert	(5, 17)
Glutamine formation	substrate	(3, 12)
Glutamine breakdown	inhibits	(3, 12)
Glutamotransferase	inhibits	(13)
	Carbamyl- $\alpha$ -methyl	
Citrulline synthesis	inert	(7)

taneously competitive inhibition may be observed for a short interval. If the  $\alpha$ -methylglutamate is allowed access to the enzyme before the glutamate, the inhibition is non-competitive.

Inasmuch as studies with deuterium show that the  $\alpha$ -hydrogen is not involved in the decarboxylation reaction (10), it is somewhat difficult to understand why the  $\alpha$ -methyl compound should not be decarboxylated, particularly since the evidence is reasonably clear that it is adsorbed to the enzyme.

With respect to transamination and dehydrogenation, where the  $\alpha$ -hydrogen is obviously involved, replacement of it by a methyl group renders the compound inert, presumably indicating that glutamic acid is adsorbed to these enzymes by positions involving the alpha hydrogen. Of course one may point out that the  $\alpha$ -methyl amino acids are incapable of forming the imino type of intermediate and if these are involved in either transaminations or dehydrogenation, an explanation is available as to why the  $\alpha$ -methyl derivatives do not react. However, the problem posed by the results is not why the  $\alpha$ -methyl substituents do not react, but rather why they do not interfere with the normal substrate. That is, an alteration in the  $\alpha$ -hydrogen apparently prevents adsorption to the enzyme. One may speculate as to the nature of the adsorption of the  $\alpha$ -hydrogen, but to the best of my knowledge there is no very certain ground for



distinguishing between several alternatives, and it is obvious that the critical experiments have yet to be conceived and executed.

In the synthesis of glutamine from glutamate, ammonia, and ATP, the  $\alpha$ -methyl derivative actually serves as alternative substrate and replaces glutamate in the system to form  $\alpha$ -methylglutamine. The  $\alpha$ -hydrogen in this case seems to be so far removed from the centers of activity and adsorption that the enzyme is not greatly influenced by the change in structure. The breakdown of glutamine, however, is inhibited by the presence of  $\alpha$ -methylglutamate. This may seem somewhat unreasonable, until it is realized that glutamate itself inhibits the reaction and  $\alpha$ -methylglutamate possesses essentially the same degree of activity.  $\alpha$ -Methylglutamate inhibits glutamotransferase. The carbamyl  $\alpha$ -methyl derivative does not interfere with the use of carbamyl glutamate in citrulline synthesis.

TABLE 2

Substance (DL)	Reaction	Effect	Ref.
$\alpha$ -Methylalanine	Bacterial growth	none	(1)
	Uptake by ascites tumor	active	(4)
	Mammalian metabolism	no effect	(11)
$\alpha$ -Methylvaline	D-amino acid oxidase	slightly inhibits methionine, valine; not phenylalanine	(17)
$\alpha$ -Methylserine	Bacterial growth	none	(1)
	D-serine deaminase	none	(9)
	L-serine deaminase	none	(9)

The effects of  $\alpha$ -methyl substitution on the metabolic reactions of some aliphatic amino acids are given in Table 2. Most are inert. However, while  $\alpha$ -methylvaline slightly inhibits the oxidation of methionine and valine by D-amino acid oxidase, it has no effect on the oxidation of phenylalanine. As shown in Table 3,  $\alpha$ -methylphenylalanine does not inhibit the oxidation of phenylalanine by the same system. These observations may serve as an approach to a better knowledge of the mechanism of action of D-amino acid oxidase, but this approach has not yet been studied experimentally.

TABLE 3

Substance (DL)	System		Ref.
$\alpha$ -Methylpyrrolidone carboxylic	glutamic decarboxylase	inert	(17)
	glutamic dehydrogenase	"	(17)
	glutamine synthesis	"	(17)
	transaminase	"	(17)
$\alpha$ -Methylphenylalanine	D-amino acid oxidase	methionine oxidation slightly inhibited valine, phenyl- alanine not.	(17)
$\alpha$ -Methyl-3, 4- dihydroxyphenylalanine	D-amino acid oxidase	weak substrate	(17)
	mushroom tyrosinase	substrate	(17)
$\alpha$ -Methyltryptophan	to kynurenine	substrate and inhibitor	(17)
	tryptophanase	inert	(9)
$\alpha$ , $\alpha'$ -dimethyl- $\alpha$ , $\alpha'$ -diaminopimelic	lysine requirement	inert	(6)

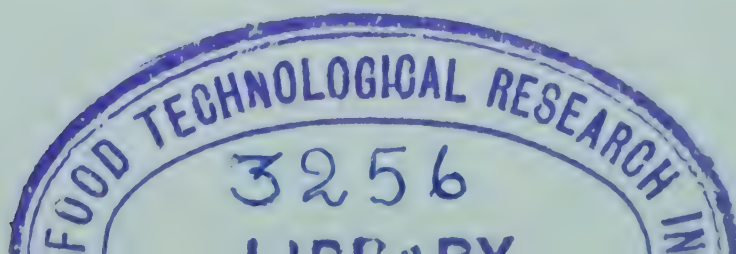
$\alpha$ -Methyltryptophan is rather interesting in that it is adsorbed and reacts with the system producing kynurenine, presumably forming  $\alpha$ -methylkynurenine, but also inhibits the transformation of tryptophan to kynurenine.

TABLE 4

DL- $\alpha$ -methyl at $10^{-3}$ M	Tyrosine decarboxylase	DOPA decarboxylase
Phenylalanine	slight inhibition *	slight inhibition
(para)-tyrosine	"	"
(meta)-tyrosine	inert	complete inhibition
methoxy-(meta)-tyrosine	"	inert
3, 4-DOPA	"	complete inhibition
3-OH-4-methoxy-PA	"	"
3, 4-methoxy-PA	—	slight inhibition

\* Complete inhibition at  $2 \times 10^{-2}$  M (from 15).

Table 4 illustrates the effect of structural alteration in other portions of the phenylalanine molecule upon the inhibitory activity of  $\alpha$ -methyl derivatives on two enzymes. On tyrosine decarboxylase,





$\alpha$ -methylphenylalanine and  $\alpha$ -methyltyrosine show some inhibition, but the other derivatives are inert, whereas on DOPA decarboxylase a free hydroxyl in the meta position is necessary before the  $\alpha$ -methyl amino acid is an inhibitor. Now this is rather curious, in that Blaschko (2) has deduced certain rules of substrate specificity for these enzymes which we have been able to extend (16). The data show that tyrosine decarboxylase requires substitution in the para position, the meta substituents being much less active, the ortho compound essentially inert. The  $\alpha$ -methyl derivatives show essentially this pattern with tyrosine decarboxylase except that the  $\alpha$ -methylphenylalanine, with no substituents, is by far the most potent inhibitor. With DOPA decarboxylase, it is the meta-hydroxy substituent which is the controlling factor for decarboxylation, and it is the meta substituent in the  $\alpha$ -methyl series which is the controlling factor in inhibitory activity.

The action of  $\alpha$ -methyl DOPA on DOPA decarboxylase was studied in some detail (15), for the reason that while at  $10^{-3}$  M it completely inhibits DOPA decarboxylase, at  $10^{-5}$  to  $10^{-7}$  M it actually increases the activity of the enzyme. This was an example at the enzymatic level of stimulation before toxicity, which is so frequently apparent in bacterial inhibition or in pharmacological experiments, and for which no satisfactory explanation has been available aside from teleological hypothesis. In fact, this type of stimulation before toxicity was perhaps the last stronghold of the vitalist.

With respect to inhibition,  $\alpha$ -methyl DOPA behaves as a slowly reversible inhibitor combining with the apoenzyme. While it reacts with pyridoxal phosphate as does DOPA itself (8, 18), the rate of these reactions is too slow to be involved in inhibition. With respect to the activation by concentrations of  $\alpha$ -methyl DOPA of  $10^{-5}$  to  $10^{-7}$  molar, various possibilities have been explored. One is that there is a second enzyme present in the DOPA decarboxylase preparation which removes an appreciable quantity of DOPA. This supposed enzyme is markedly inhibited by  $\alpha$ -methyl DOPA, hence low concentrations of  $\alpha$ -methyl DOPA reduce spurious loss of substrate from



the decarboxylation and thus apparently stimulated it. However, DOPA lost can all be accounted for as  $\text{CO}_2$  released, so that it is certain that there is no second system present which is capable of using rather large quantities of DOPA.

One may suppose that  $\alpha$ -methyl DOPA increases the effective amount of coenzyme by "protecting" the pyridoxal phosphate against the sequestering action of the substrate. Coenzyme concentration studies show that this hypothesis is not tenable, but perhaps the best argument against it is that  $\alpha$ -methyl-3, 4-dihydroxyphenylalanine also shows a corresponding activation of the decarboxylation of 2, 5-dihydroxyphenylalanine, a compound which has no discernible reaction with pyridoxal phosphate and thus no sequestering action in this respect.

Another possible explanation for the stimulation by  $\alpha$ -methyl DOPA is that this substance, like other hydroxyphenylalanines (2), occupies binding sites on the apoenzyme by way of its phenolic groups, and that some of these binding sites are not associated with decarboxylation. If it be assumed that  $\alpha$ -methyl DOPA has a greater affinity for such sites than for the binding sites of the active centers, it might thus promote decarboxylation by blocking off competitive sites on the enzyme surface to which DOPA might be bound but at which no decarboxylation would occur. At the moment we know of no way of either proving or disproving this hypothesis, at least with the DOPA decarboxylase preparations available today. It therefore remains a possible or even probable hypothesis; but only because we cannot subject it to experimental test.

We feel that the preceding examples demonstrate the potential value of amino acids substituted in the alpha position for the development of a more detailed knowledge of enzymatic reactions and pathways.

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#### DISCUSSION

DR. UDENFRIEND: I would like to ask Dr. Meister one general question. Is the mechanism of amino acid oxidation generally considered to involve an intermediate imino compound?

DR. MEISTER: I think the best that we can say is that the available data are compatible with this interpretation which demonstrates the formation of an imino acid as an intermediate. The data that Dr. Umbreit just presented on alpha methyl amino acids are of interest in this connection. The alpha methyl amino acid derivatives are incapable of being oxidatively deaminated or of entering into transamination reactions.

DR. UDENFRIEND: Would anybody care to comment on imino compounds?

DR. HANDLER: I would like to speak to that point. About 15 years ago we published a group of observations which is relevant to this problem and also the problem which Dr. Meister raised with respect to what the significance of D-amino acid oxidase in animal tissues might be. Valine, leucine, and isoleucine are in vitro excellent substrates for D-amino acid oxidase—both in purified systems, homogenates, and slices. These 3 amino acids when given to the intact animal in the D form are excreted in large quantities, in contrast to the D form of phenylalanine, for example, which does not appear as such. These 3 amino acids, which are excellent substrates for the D-amino acid oxidase, can, however, be replaced by the keto acid for the growth of the rat, but in comparable quantity these 3 D-amino acids will not replace the L-amino acids in the diet of the rat, whereas L-phenylalanine, tryptophane, and so forth will replace them. This problem has



puzzled us considerably. There is also a correlation here whose significance is even less clear, namely, the  $\alpha$ -N-methyl derivatives of the L-amino acids whose D-forms can be used by the rat for growth are also available to this species. However, the  $\alpha$ -N-methyl derivatives of L-valine, leucine and isoleucine, whose D forms are relatively unavailable are also not utilized by the rat for growth. At the time, we considered the possibility that D-amino acid oxidase in vivo was not operating the same way in which it is observed to operate in vitro and the further possibility that there was an alpha-beta desaturation in the initial reaction which could be blocked by the methyl substituents of these three amino acids. In other words, the initial step might not be an imino acid but an alpha-beta desaturation.

There is, of course, the possibility that all of these data reflect, not the hepatic metabolism of these amino acids but the ability of the rat kidney to retain them within the animal.

DR. MEISTER: May I take slight issue with you on one point. It has been reported that D-valine will replace L-valine in the diet of the rat. It has to be used at a somewhat higher level than L-valine. Furthermore, I believe that some of the earlier isotope work indicated that the carbon of D-leucine can be converted to L-leucine in the rat, at least to some extent. It would seem that there is some evidence that D-amino acid oxidase attacks these amino acids in vivo, although apparently not rapidly enough to permit them to completely replace the L-isomers in supporting growth. The alpha-beta desaturation concept is a little difficult to see in view of the data which have been obtained on oxidation and transamination.

DR. ADELBERG: I just recall von Euler's suggestion on glutamic acid in this respect. He suggested that an imino acid was an intermediate because of spectrophotometric changes which he observed during the course of the reaction. I wonder if anything further can be made of that.

DR. STRECKER: Someone has already raised the question concerning the formation of imino glutaric acid as an intermediate in the deamination of glutamic acid. Both Olson and Anfinsen and ourselves performed some experiments to verify the formation of this compound, and we could get no evidence whatsoever for such a compound being formed. Von Euler assumed that the first step in the oxidation of glutamic acid results in imino glutaric acid and reduced DPN. Then he assumed that the second stage was a nonenzymatic hydrolysis of the imino glutaric acid to form alpha keto glutaric acid and ammonia. This would presumably be, according to von Euler's concept, a truly reversible reaction and also a nonenzymatic one. If this were true one would assume that the formation of imino glutaric acid would be proportional to the product of the concentrations of alpha keto glutaric acid and ammonia. If one were low, then by raising the other one could get the same formation of imino glutaric acid as if the other were



low and the first were raised. We found that this was not true. We found that each of these entities had a definite affinity for the enzyme independent of the concentration of the other.

DR. LIPMANN: I would like to mention briefly this general point on the slow activation of enzymes by the pyridoxyl amine phosphate. We have observed this same effect in pyruvic oxidase when FAD is added. I think that when we investigate this phenomenon further we will find that in general it will take considerable time for reactivation of enzymes to occur, particularly when the coenzyme dissociates from the apoenzyme with some difficulty.

DR. GUNSALUS: The point you are making is, I believe, certainly true for pyridoxal phosphate—namely, that the concentration of the enzyme is also important with respect to the time required for complete activation.

DR. MEISTER: We cannot really distinguish at the present time between a pyridoxamine phosphate-decarboxylase and pyridoxamine phosphate combined with another protein in the enzyme preparation. We don't know where the pyridoxamine phosphate is bound, but we believe that it must be tightly bound to a protein.

DR. COHEN: I think that Dr. Umbreit has encouraged this group to pay more attention to the properties of pyridoxal phosphate in order that we might obtain further information on its properties for comparison with the material which Merck is so generously handing out. Dr. Brandenburger in our laboratory obtained some crystalline pyridoxal phosphate from Professor Karrer and coworkers and compared it with the material that Merck is supplying. If one uses the resolved glutamic-oxaloacetic transaminase as prepared by O'Kane and Gunsalus and compares its reactivation by the crystalline pyridoxal phosphate with the material supplied by Merck (which is stated to be 75% to 80% pure), the Merck preparation is anywhere from  $1\frac{1}{2}$  to 3 times as active as the crystalline pyridoxal phosphate. I think that this can mean only one thing—namely, that the Merck preparation has something in it besides pyridoxal phosphate which is useful to the enzyme system. We, like so many others, have been stimulated by the work of Snell and co-workers and are looking into trace metals, but we have not as yet put our finger on anything that is particularly revealing. It might be of interest to point out that the emission spectrum of the Merck preparation shows a strong Fe band. I feel that Dr. Umbreit is correct in saying that a closer examination of the pyridoxal phosphate should be made and that present evidence does not preclude other factors being essential.

DR. MEISTER: I would just like to add my few cents' worth to this discussion, since Dr. Peterson and Dr. Sober in our laboratory have prepared crystalline pyridoxamine phosphate and pyridoxal phosphate, and we have compared it with samples supplied by Merck. In our hands the preparations



from Merck varied from 30% to 50% purity based on our present methods of assay. The crystalline material assayed 100%, while the samples obtained from Merck and Company gave values which confirmed the Merck assays. We will agree that there is something in the Merck preparation which is not pyridoxal phosphate, but we don't attribute any importance to this extra material in terms of enzymatic activity.

DR. UMBREIT: I think the alternative hypothesis is that there might be two things in the crystalline pyridoxal phosphate. Suppose, for example, that during the crystallization one actually obtained what might be considered 2 isomers of the compound. I don't know what they would be. If the crystals possessed, however, just half the activity, then it might be that the non-crystalline material would be more active than the crystalline product.

DR. GUNSALUS: Dr. Cohen, have you tried any of Dr. Meister's material?

DR. COHEN: No, we have not.

DR. TANENBAUM: With regard to pyridoxamine-keto acid transamination, I would like to unburden myself of a datum and bring this topic up for discussion. About 5 years ago, when it was still fashionable to use  $N^{15}$ , Dr. Shemin and myself succeeded in synthesizing labelled pyridoxamine and we were to show then that it transaminated very nicely in a pig heart system with alpha keto glutaric acid to give L-glutamic acid. Ammonia was not an intermediate in this process. As Dr. Umbreit points out, the question arises as to whether the Snell hypothesis accounts for all the facts. I should expect with this formulation, that pyridoxamine will be capable of entering into transamination with all the keto acids. As far as I can tell, there are only a few isolated instances in the literature of actual transaminations with pyridoxamine or its derivatives as substrates. I think Dr. Meister and Dr. Gunsalus have had several, and I wanted to add this pyridoxamine-alpha keto glutarate case to the roster. Incidentally, it was not necessary to add ATP nor any of the phosphorylated compounds to the system to get effective transamination. I don't know whether the amine was phosphorylated before entering into the reaction, or whether the phosphorylated coenzyme was already on the enzyme and the pyridoxamine was being handled just as any other amino donor.

DR. GUNSALUS: It was in fairly high concentrations?

DR. TANENBAUM: Yes.

DR. ROBERTS: I would like to ask Dr. Meister which of these various transamination enzymes he thinks are important in the intact cell and whether he would like to say anything about the point of entry of ammonia into cells growing with glucose as the sole carbon source and without any additional amino acids.



DR. MEISTER: I would like to beg off on that question, since our work has been done with isolated enzymes. I will have to leave to others the answer to that question.

DR. SPRINSON: With regard to the possibility of  $\alpha,\beta$ -dehydrogenation as a stage in amino acid metabolism it is worth mentioning the experiments which Dr. Rittenberg and I did some years ago with  $\alpha,\beta$ -deuterio-L-leucine. We could find no evidence for such a reaction *in vivo*.

I should like to comment briefly on the role of pyridoxamine phosphate in transamination. In an investigation carried out in Dr. Rittenberg's laboratory by Dr. Peyser on the exchange of the  $\alpha$ -hydrogen of amino acids under the influence of transaminases it was shown more than two years ago that pyridoxamine phosphate was as active as pyridoxal phosphate in exchange of the  $\alpha$ -hydrogen of glutamate by a purified, completely resolved pig heart glutamic-aspartic transaminase. Both coenzymes were Merck preparations. Very small concentrations of  $\alpha$ -ketoglutarate were necessary for maximum activity, but high concentration of keto acid prevented maximum exchange.

May I ask Dr. Meister one question? Does the appearance of glutamic acid following the incubation of a crude enzyme preparation with an amino donor and  $\alpha$ -ketoglutarate necessarily mean that *direct* transamination has taken place? Actually some other transformation may have occurred first.

DR. MEISTER: I would certainly agree with you, Dr. Sprinson, about the necessity of investigating all the reactants in transamination reactions. This point has been made several times, for example, Herbst, complained that enzyme chemists tended to use shortcuts and recommended that the products of a reaction be isolated. This is one of the things we have tried to do in our experiments. I think the experiments that you were referring to were carried out with crude preparations of animal tissues in which a number of amino acids were incubated with  $\alpha$ -ketoglutarate. Under these conditions the formation of glutamic acid does not unequivocally show direct transamination between keto glutarate and the amino acid. An example of such a situation is the case of transamination involving arginine, where the presence of arginase in the crude preparations unquestionably converted the arginine to ornithine. With regard to your question about glycine, I do not recall any experiments on glycine transamination in which glyoxylate was isolated as a product. Unless one knows the configuration of the resulting carbon chain one cannot be sure that a direct transamination has occurred.

DR. COHEN: I would go along with that completely; and, unless one has a purified system from which the various products can be isolated and identified, no definite statements with respect to the nature of the transamination can be made. Balanced experiments must be performed in all cases.

DR. SINGER: I would like to add a bit of information with respect to the



activity of the Merck pyridoxal phosphate and that obtained from other sources. While working with highly purified preparations of pyridoxal kinase, Dr. J. Hurwitz had occasion to compare the activity of various synthetic Merck preparations of pyridoxal phosphate (kindly supplied by Dr. Umbreit) with the enzymatically synthesized product. Dr. Hurwitz has shown (*J. Biol. Chem.* 205, 935 (1953)) that the action of the kinase involves the disappearance of 1 mole of ATP for each mole of pyridoxal phosphate formed. Thus the concentration of phosphorylated pyridoxal could be *independently* estimated by 3 methods: disappearance of labile phosphate, the tyrosine apodecarboxylase test, and spectrophotometry. Using these techniques, Dr. Hurwitz found no evidence for a higher activity of the Merck product in the *Strep. faecalis* tyrosine apodecarboxylase test than of the enzymatically synthesized coenzyme.

It may be pointed out, however, in view of Dr. Cohen's remarks, that some methods of estimation of pyridoxal phosphate (including the tyrosine apodecarboxylase test) involve the use of whole organisms or of crude enzyme preparations, whereas others utilize extensively fractionated enzymes. Thus it is conceivable that a trace metal effect might show up in one assay and not in another.

DR. STRECKER: Dr. Alex Keynan in our laboratory has recently obtained some experimental results which reveal a hitherto unsuspected relationship between amino acids and glycolysis. Using washed suspensions of *Bacillus subtilis* it was found that glycolysis was stimulated by the addition of either glutamine or glutamic acid as demonstrated in Fig. 1. The stimulation was

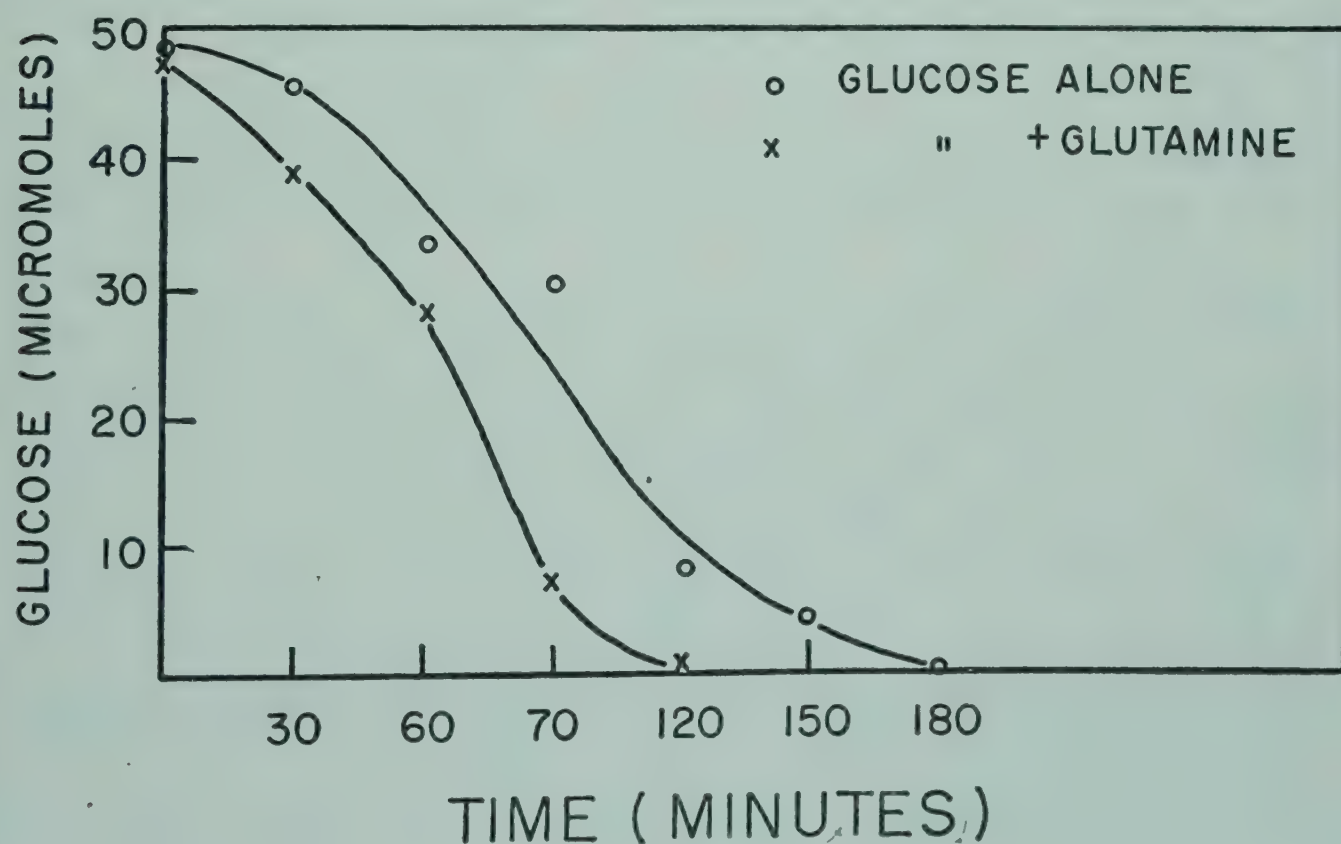


FIG. 1.



TABLE 1

PRODUCTS OF GLUCOSE METABOLISM WITH AND WITHOUT GLUTAMINE ADDED

	Exp. I		Exp. II	
	pH 7.4		pH 6	
	240 min.	240 min.	240 min.	240 min.
	$\mu M.$	$\mu M.$	$\mu M.$	$\mu M.$
Glutamine added	0	10	0	10
Glucose used	47	47	13.7	26.9
Acetoin formed	15.2	25	7.2	19.9
2 : 3 butandiol	2	0	—	2.1
Lactic Acid	5	7	—	—
Steam volatile acids	57	36.7	17.5	4.6

Total volume 6 cc. 35° C. Time of experiments 240 min. Every 2 cc. contained 50  $\mu M.$  of glucose. Phosphate buffer  $M./15$ . The values given are on the basis of 2 cc.

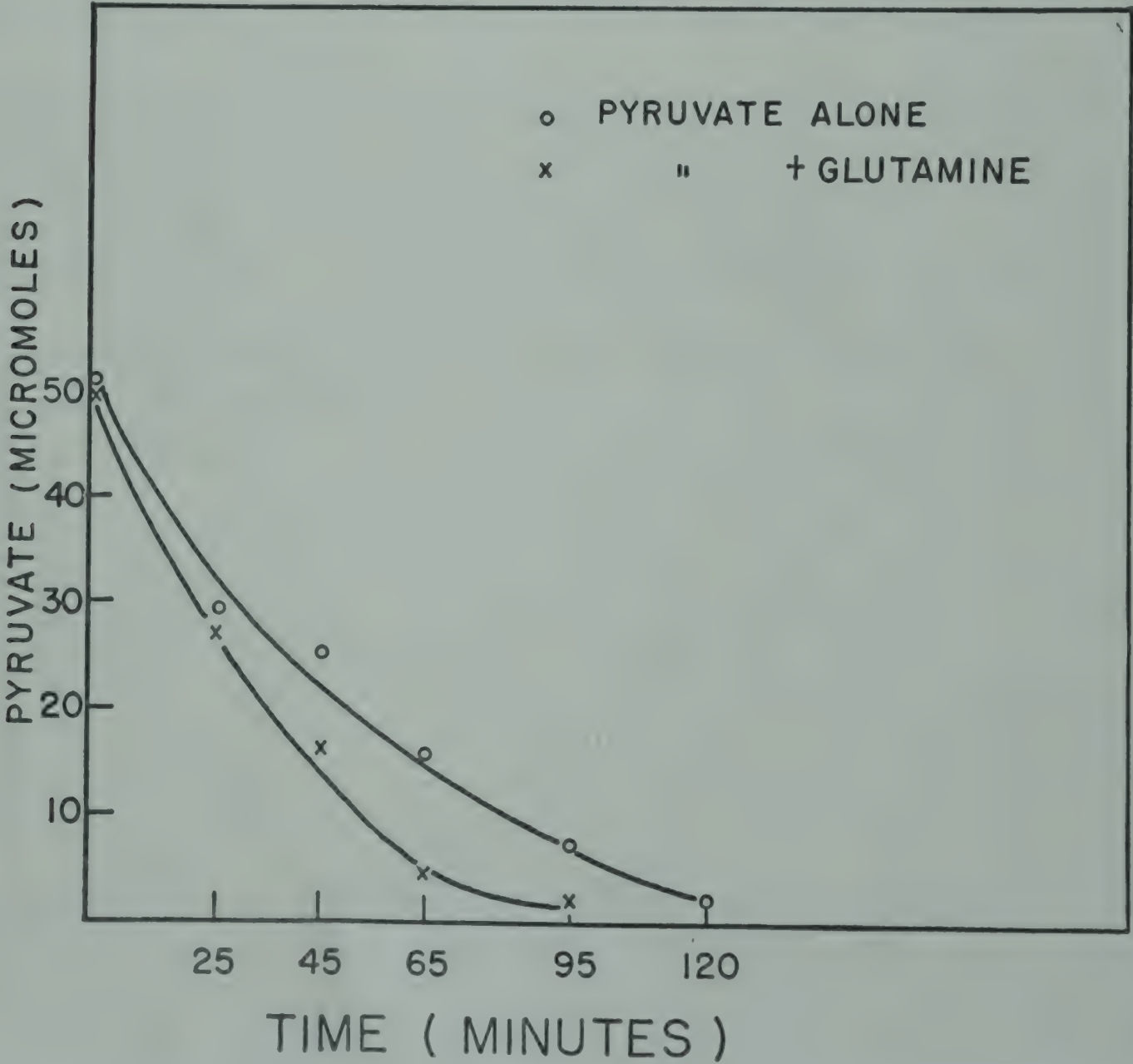
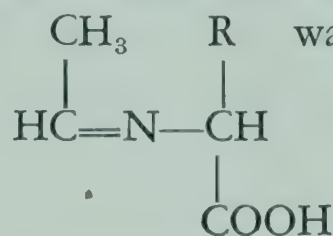


FIG. 2.

found to be proportional to the amount of amino acid present and in the range of concentrations studied up to a three fold increase in the disappearance of glucose could be obtained.  $\alpha$ -Ketoglutarate on the other hand does not stimulate but rather markedly inhibits glycolysis. Analysis of the products of glucose metabolism in this system revealed mainly acetoin and steam volatile acids. The addition of either glutamine or glutamic acid increased the formation of acetoin and decreased the proportion of steam volatile acids. The results of some experiments are shown in Table 1. Repeating the study on the level of pyruvate resulted in the same picture, i. e. an increase in pyruvate dissimilation, an increase in acetoin and a decrease in volatile acids on the addition of either glutamine or glutamic acid. The effect of glutamine addition on pyruvate disappearance is shown in Fig. 2.

We are exploring the possibility that the effect of these amino acids on the formation of acetoin may be related to the classic observations by Herbst on the reaction between pyruvate and  $\alpha$ -amino acids in which the formation of an intermediate aldimino compound



It is possible that a similar compound is formed in the *B. subtilis* system which can react either with an active acetaldehyde derived from pyruvate, or with pyruvate itself.

DR. DEMOSS: I would like to ask Dr. Strecker how the organisms were grown. There seem to be some marked differences in the pathways utilized by the organism, depending upon whether it was grown in a simple medium or a highly complex medium.

DR. STRECKER: This was a washed cell preparation which was grown on Santon's media containing citrate, glycerol, salts and glutamate in a Roux bottle.

DR. HANDLER: I would like to add one bit of information which gave us pause in considering the nature of the steady state with respect to hepatic nitrogen metabolism. Dr. Meister has clearly described the diverse pathways now known to be available to glutamine and the apparent nature of the highly active 'glutaminases' of liver from which one would infer that the glutamine of liver should be in equilibrium with such  $\text{NH}_3$  as exists there and the latter in turn, by virtue of the glutamic acid dehydrogenase, should be in equilibrium with the glutamic acid pool. To test this, we gave glutamine, labeled with  $\text{N}^{15}$  in the amide position to pigeons and at the same time gave them sodium benzoate. Uric and ornithuric acids were then isolated from their subsequent excreta and examined for  $\text{N}^{15}$ . The uric acid accounted



for almost 90% of the isotope administered and its  $N^{15}$  concentration was almost 30 times as high as that of the ornithuric acid. Since both nitrogens of ornithine appear to be derived from glutamic acid, it would seem that, in the pigeon liver, either the hydrolysis of glutamine is trivial in the steady state, or once hydrolyzed the only significant pathway of the ammonia is back to glutamine rather than to glutamic acid. These data appear to provide an *in vivo* confirmation of Buchanan's observation that two of the purine nitrogens are directly derived from glutamine. We also suspect that they may well constitute a warning that 'glutaminase' activity may be more apparent than real and that, like ATPase activity, may represent the activity of an enzyme system whose proper transfer function has not yet been established.

# MODE OF TRANSPORT OF AMINO ACIDS INTO CELLS

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## INTRODUCTION

THE FIRST EVENT in the cellular utilization of an amino acid is its transfer into the cell interior. The existence of such a special process was discovered in 1912 by Van Slyke and Meyer (1). They showed not only that tissues were 5 to 10 times as rich in apparently free amino acids as the plasma, but also that increases in the plasma amino acids were mirrored by larger increases in the tissue amino acids. Subsequent study of this behavior by Luck (2), Hamilton (3), and Christensen et al. (4-7) has shown that this process exists for numerous individual amino acids, as well as for the amino acids collectively. Many species have now been shown to possess apparently similar cellular activities.

The cellular amino acids are not merely fixed at a level higher than those of the plasma; instead they rise and fall with the extracellular level. This behavior is particularly striking for glycine in the fasting guinea pig, because the amount of this amino acid present in the free state shows a wide spontaneous variation. These variations were found, however, to take place together for the plasma, the liver, and the muscle (5). This behavior could also be demonstrated with the excised diaphragm of the rat by manipulating the extracellular glycine level upwards and downwards (6). In general, the amino acids of cells of the higher animals have been found not only to rise but also to fall with the extracellular level. In order to express the concept that the extracellular and cellular levels of the amino acids are reactants upon which the direction of net transfer depends, the process has been referred to as the *concentration* of



amino acids. The behavior observed appears to be somewhat different from the accumulation of amino acids by certain gram-positive bacteria, as studied by Gale and his associates, and recently reviewed (8). These microorganisms are able to hold the accumulated glutamic acid upon washing in distilled water, and they release the glutamate only under special metabolic circumstances. In fact the method of investigation includes only accumulated amino acids which are retained upon washing the bacterial cells. The expectation that the transfer process nevertheless would be found to proceed by similar reactions in bacteria and higher animals has not yet been realized. For cells of higher animals the amino acids appear from several lines of evidence to be largely in the free state within the cell; the process is therefore an active transport. The behavior in some bacterial species, on the contrary, has led investigators to question whether the process under study is one of active transfer into the cell (8).

#### SIGNIFICANCE IN THE CONTROL OF CATABOLISM AND GROWTH

The striking feature of amino acid metabolism in the higher animals is the large variation in the proportions of the incoming amino acids which on one hand are destroyed and which on the other hand are retained. The change of these proportions from infancy to adulthood is very large, but extremely important changes are also induced by injury, diseases, or hormone administration. This is also the central problem of amino acid nutrition, because the amino acids must be supplied under conditions which will permit adequate anabolic utilization.

The distribution of each amino acid in the fasting animal appears to be under two principal influences:

- (1) the cellular activity by which cells transfer the amino acid into their interior; and
- (2) the reactions within the cells which utilize or form the amino acid.

The relationship between these two factors can be illustrated by the two amino acids, glycine and leucine. Leucine is found at



comparatively high levels in the plasma of certain animal species. The explanation is that the process by which leucine is transferred into cells against an apparent gradient is comparatively ineffective, and therefore a substantial extracellular leucine level will necessarily exist to "back up" the cellular levels, which in turn are in a steady state with cellular reactions. Glycine is also very abundant in the plasma, but in contrast with leucine this amino acid is very strongly concentrated by the cells. Here the high level outside the cells is explained by the very high cellular levels which exist in a steady state with the reactions which form and destroy glycine.

Because the reactions which destroy amino acids are not uniformly distributed through our cells, but occur predominantly in certain tissues, especially the liver, modification of the proportions catabolized can occur not only by direct effects upon degradative reactions, but also by modifications of the cellular transfer processes. For example, a decrease might be produced in the levels of the amino acids exposed to degradative enzymes in liver cells, with the result that larger quantities of the amino acids are available elsewhere for synthetic reactions. In addition, particularly high levels may be produced in one tissue, so as to facilitate synthetic reactions at this site, and thereby decrease utilization at other points. Small changes in distribution over an extended period of time could produce large differences in growth.

The pregnant animal serves as a good illustration. Here we find that amino acids are transferred concentratively from the maternal to the fetal circulation, across the placenta (9). Concentration by 1.5 to 5 times may occur, according to the species. The depression of the amino acid levels seen for the maternal plasma (10) is undoubtedly a consequence; the fetal cells in contrast live in an enriched medium. In the rabbit a further enrichment occurs due to especially active transfer into the fetal cells. A number of other examples of enriched amino acid environments associated with accelerated growth has been observed.

The concentration achieved by the transfer process averages for the whole animal less than ten times the extracellular level, if it is assumed that the cellular amino acids are distributed throughout



the cellular water. Unless the amino acids are delivered at much higher local concentrations, the process does not represent a large part of the energy of protein synthesis, inasmuch as proteins can spontaneously release amino acids to very high concentrations in the presence of proteolytic enzymes. For cells which exist in very dilute amino acid solutions, however, the accumulation process may represent a much larger part of the energy requirement. The rather limited exploration so far carried out suggests the stronger operation of the transfer process in the nutrition of primitive organisms. In the higher animal the process appears to be restrained and may have more importance in the control of amino acid catabolism and growth than in facilitating protein synthesis energetically. Nevertheless, with so many reactants involved in the synthesis of a protein molecule, a concentration by as much as ten times obviously can have a large effect upon the rate.

*Significance as reserve precursors for protein synthesis.* The synthesis of protein requires the presence during definite intervals of time of each amino acid that is to be built into the macromolecule. Delay in the supplying of one amino acid can be tolerated only to the degree that the other amino acids can be retained without destruction. The apparent absence of comparable accumulations of other precursors of small or intermediate molecular size gives the cellular accumulations of amino acids special significance as reserve precursors for protein synthesis.

*Significance in the transfer of amino acids from one extracellular phase to another.* The concentrative nature of the transfer of amino acids into the fetal circulation has been mentioned. The studies of Wiseman et al. (11-13) and of Agar, Hird, and Sidhu (14) emphasize that transfer across the intestinal mucosa also occurs against a gradient, as must also the reabsorption of amino acids by the renal tubules. All that is required to permit a cell-layer to act in this way is that the concentrative process be weaker on one surface than on another. Conceivably such cells instead may have their own distinct transfer apparatus, but a priori this appears less likely.

*Susceptibility of the concentrative process to competitive inhibition—amino acid imbalance.* Whenever any of several amino acids fed to the guinea pig succeeded in producing high plasma levels, the transfer of glycine into cells was interfered with, so that the liver and muscle glycine fell and the plasma glycine rose (Fig. 1). This was interpreted as a competition for the concentrative process.

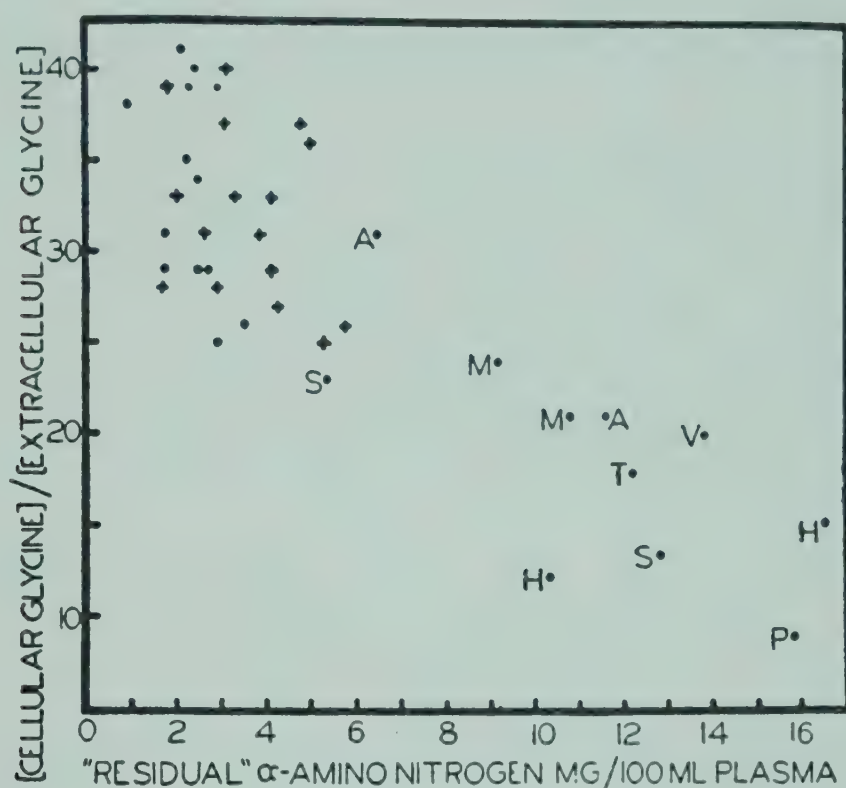


FIG.1. Showing that the elevation of the plasma amino acids of the guinea pig by feeding a single amino acid decreases the ability of the liver to concentrate glycine (5). ●, fasting controls; +, experiments with various amino acids which increased the plasma amino acids only slightly; A = DL-alanine, H = L-histidine hydrochloride; M = L-methionine; P = L-proline; S = DL-serine; T = DL-threonine; V = DL-valine. (From Christensen, H. N., Streicher, J. A., and Elbinger, R. L., *J. Biol. Chem.*, 172, 515, 1948).

Subsequent study with isolated cells has verified the susceptibility of this process to competition, undoubtedly because of its low specificity. The transfer process is a likely site for the production of the harmful nutritional effects of unbalanced combinations of amino acids; an excess of one amino acid may thus bring other amino acids to very low levels in the cells. In agreement, Kihara and Snell (15) have shown that if the level of L-alanine is not kept low in the culture medium of *Lactobacillus casei* this amino acid interferes with the cellular uptake of the essential D-alanine.



## I. SELECTION OF TISSUE FOR STUDY IN VITRO

*Tissue Slices.* The release of proteolytic activity when a tissue is sliced is a serious disadvantage to the study of amino acid distribution by means of slices. The rapid increase of the extracellular amino acid levels led us to turn instead to the diaphragm. Stern, Eggleston, Hems, and Krebs (16) found, however, that an accumulation of glutamate could be observed with slices of the cerebral cortex of the guinea pig, a gradient of about 23 millimoles per kilogram being established between the tissue and the suspending fluid. Accumulation was shown also in kidney cortex, spleen, lung, and chorion. The presence of oxygen and of glucose, fructose, L-lactate, or pyruvate was necessary to support this activity. During these experiments a substantial net loss of glutamate occurred. The efficiency of the process decreased rapidly after 60 minutes.

*Diaphragm.* Concurrently the transfer process of the isolated rat diaphragm for glycine was investigated (6). Very little production or consumption of glycine occurred with this tissue. The glycine content of the tissue could be increased or decreased at will by manipulating the glycine level of the suspending fluid, the level calculated for the cell water remaining about 7 times that in the medium. The process of transfer into the cells was highly temperature-sensitive; lowering the temperature changed glycine entrance *against* the gradient to glycine exit *with* the gradient. The exodus of glycine on the other hand had a  $Q_{10}$  of about 1.2 and probably occurred by diffusion. The glycine could be reaccumulated from the suspending medium upon rewarming the diaphragm. Pyruvate but not glucose stimulated accumulation; oxygen was necessary, and 2,4-dinitrophenol and cyanide were strong inhibitors. Here the inhibition of amino acid uptake by the potassium ion was first noted. Inhibitions among the amino acids were also noted (7).

The diaphragm of the rat is in many ways a very convenient tissue for this study, but progress is greatly hampered by the necessity of working with a very thin diaphragm, and therefore a small mass, permitting only limited subdivision. Furthermore, in recent studies



we have found that the diaphragm loses about half of its potassium in one hour in an otherwise suitable medium. The indications are that with the diaphragm as with tissue slices, the interior of the tissue is not optimally maintained during incubation. To be fully suitable for these studies a tissue should be maintainable in a suitable medium *in vitro*, so that only small shifts in electrolytes and water occur. This criterion follows from the close relationship of amino acid transfer to potassium transfer, to be discussed below.

*Erythrocytes.*<sup>1</sup> The mammalian erythrocyte has a rather slight ability to concentrate amino acids. The avian erythrocyte, in agreement with its greater respiratory and anabolic activity, is much more active in this respect (17). The reticulocyte of the rabbit has a similar degree of activity, but this is lost upon maturation (18). The process in erythrocytes in general is scarcely inhibited by cyanide or 2,4-dinitrophenol; the energy for the process presumably arises largely from glycolysis rather than from respiration. The transfer is, however, readily inhibited by elevations of the potassium ion concentration.

The atypical nature of the process in the erythrocyte may be looked upon as a disadvantage to investigation with this cell; on the other hand if the difference is in the delivery of energy and not in the transfer process itself, the use of the erythrocyte may assist in the dissociation of direct effects on the transport process from effects upon energy-delivering reactions.

*Free neoplastic cells.* The biochemical utility of another type of tissue has been emphasized by Klein (19). This is the free-cell neoplasm. Many different types of these tumors have been produced by the intraperitoneal injection of cell suspensions derived from solid tumors. The multiplying cells stimulate a voluminous transudation into the peritoneal cavity, and the cells show no tendency to cohere but remain in free suspension. Cell division may occur as rapidly as every 3 hours. The nutrition of these cells presents

<sup>1</sup> Of the following experiments, those originating in the reviewer's laboratory have been supported in considerable part by a grant (No. C-1268) from the National Cancer Institute, National Institutes of Health, United States Public Health Service. Assistance has also been received from the Abbott Laboratories, Inc.



many interesting aspects. One of these is the necessity of the very rapid increase in the volume of the extracellular fluid of the host. In the growth of the Ehrlich mouse ascites tumor (20), the amino acids appear to enter the peritoneal cavity by diffusion and then are captured by the neoplastic cells. These outdo the cells of the host in accumulating an administered dose of glycine or alanine (21).

The rate of multiplication of these cells *in vivo* is so fast that they should exhaust the free amino acids contained in them and in the ascitic plasma in less than an hour. After removal of the cell suspension from the host, however, the disappearance of free amino acids continues only briefly. In agreement with this, no new mitoses are observed after the collection of the ascitic fluid. The nature of the dependence upon the host which terminates growth is one of the fascinating problems presented by these cells. Reimplantation of fresh cells into a mouse peritoneal cavity in a thinned suspension in a collodion bag (with 1 ml. of cell-free ascitic fluid placed free in the peritoneal cavity) permitted an increase from 13 million to 26 million in 42 hours (22). The dry weight of the cells was, however, not increased, so there was no growth in the chemical sense. Under these conditions the cells showed a tendency to clump. This result is not taken to indicate that macromolecules are required for the nutrition of the cells; other possible explanations for the failure of growth are the damping by the collodion bag of the convection currents ordinarily serving to agitate the cell suspension, and the exclusion of a possible host factor ordinarily serving to prevent cohesion of the cells.

Although we would like very much to know why these cells cease to grow *in vitro*, as far as the study of amino acid distribution is concerned the near cessation of anabolic utilization of amino acids represents an advantage. Amino acid catabolism is also small. Very little loss or gain occurs during incubation of the cells with various amino acids, except that substantial losses are observed with glutamine, arginine, serine, and ornithine: the loss of arginine is accompanied by increases in ornithine and urea; the loss of serine, by increases in glycine; and the loss of ornithine, by increases in alanine (23).



The Ehrlich ascites tumor cells show a high endogenous respiration, continuing unabated for at least 4 or 5 hours. They still produce the ascites tumor upon reinoculation after several days of refrigeration at 5° C., and remain viable after quick freezing (24). The cells are almost completely resistant to rupture in the Potter-Elvehjem homogenizer. Although the Krebs' Ringer-bicarbonate medium is not quite correct in composition to maintain precisely the potassium and water content of the cells, only small shifts are shown, and there are no indications of deterioration of the cells during incubation. In short, the Ehrlich ascites carcinoma cells appear to be highly advantageous for the purpose of studying the transfer process, in that they show a very high concentrative activity for amino acids and are readily handled *in vitro* without injury. Furthermore, they are available in abundance, and in suspension can be sampled very accurately. A slight inconvenience is the necessity of centrifugation to collect the cells, but it has been found possible to minimize changes during this procedure.

## II. IMPLICATIONS OF THE METHODOLOGY ON THE STATE OF AMINO ACIDS IN THE CELLS

The apparently free amino acids are released from the mouse-ascites carcinoma cell by a variety of procedures, most frequently by extraction with saturated aqueous picric acid. They are also obtained in the extracellular phase by washing the cells with any medium which is not adequate to support the concentration process, e. g., an adequate volume of any amino-acid-free solution, or a solution containing cyanide or 2,4-dinitrophenol, or any aqueous solution in the cold, or in the absence of oxygen. In the case of the diaphragm the absence of pyruvate or an equivalent substrate causes the appearance of the accumulated glycine in the medium. Extraction with alcohol to a 70% concentration also yields the amino acids in forms which show the characteristic chromatographic behavior of the free amino acid on paper. Of the analytical procedures used, chromatography appears to be the mildest. Numerous workers have now shown the presence of chromatographically free amino acids



in various tissues. Some of the methods we have used would include amino acids released from any soluble combinations unstable at 100° C. in nearly neutral solutions; but the results obtained in general are in agreement with those by milder procedures.

Two standards of reference have been used in expressing the amino acid uptake: (a) for the rate of uptake, the millimoles of amino acid per unit of dry weight of cells; and (b) for the levels reached in the cells, either the same designation (Stern et al. (16); Gale, see ref. 8 for bibliography) or the number of millimoles per kilogram of cell water. The latter mode of designation has proved very intelligible for the cellular inorganic electrolytes, for example, potassium. It is not necessarily assumed that the accumulated amino acid is distributed in the cell water, although strong indications have been obtained that the barrier which separates high and low potassium levels in the mammalian cell is the same one which separates high and low amino acid levels.

Since the carcinoma cells are normally 80 per cent water, the designation based upon the weight of cell water can be converted to that based upon dry weight by multiplying by four, except that in some cases swelling or shrinkage of the cells must be taken into account.

### III. EXTENT AND RATE OF THE CONCENTRATION PROCESS

*Steady state distribution.* The relationships between the external and the internal amino acid concentration at an approximately steady state are shown in Fig. 2, for glycine in avian erythrocytes, and for glycine and tryptophan in the ascites tumor cell. Most of the transfer of added glycine took place within the first 15 minutes, although 1 to 3 hours of incubation were actually allowed. As the amino acid level of the suspending fluid is raised, the relation is at first curvilinear, until a maximum gradient is reached, which tends to be maintained constant with further increases of the extracellular glycine. A similar tendency was noted in the accumulation of glutamate plus glutamine by slices of cerebral cortex (16), although the bacterial studies (25) showed that very little further glutamate



could be made to enter the cells after a certain extracellular level (roughly 0.5 millimolar) had been reached.

If the gradient really were precisely constant, this would argue against the transfer of the amino acid from one level to a higher one, both in the free state, and in favor of the entrance of the amino acid into the major portion of the cell water by diffusion and the conversion of a portion into an unstable bound form. In this situation, as soon as the binding groups were saturated a further

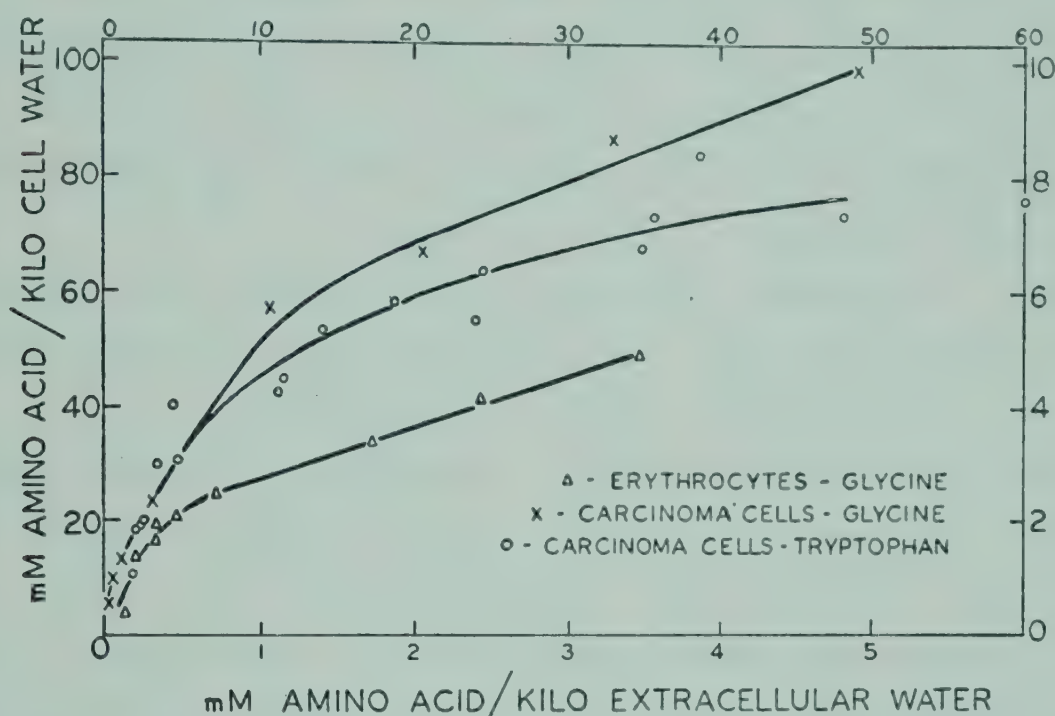


FIG. 2. Steady-state distribution of amino acids between suspending fluids and duck erythrocytes or Ehrlich ascites carcinoma cells. The abscissa scale at the bottom and the ordinate scale at the right refer to the erythrocytes, the scales at the top and left to the carcinoma cells.

(From ref. 17, 18, and 41).

increase of the extracellular level should be paralleled by an equal increase in the level in the cell water. Inversely, the failure of the cellular glutamate of *Staphylococcus aureus* and of *Streptococcus faecalis* to respond to increases of the external glutamate from 0.6 millimolar to 10 millimolar seems to argue against the diffusion of glutamate into any major part of the cell water. This is mentioned because data obtained by Britten have been interpreted as indicating that glutamate does diffuse into about half of the cell water of *Staph. aureus* (8).

The relative constancy shown by the gradient does not, however,



establish the process to be a simple binding of the amino acids diffusing into the cell, but may be explained in other ways. First of all, a gradual decline in the distribution ratio with a rising amino acid load would give a curve (as in Fig. 2) tangential to a line describing a constant gradient, perhaps over a long interval, so that considerable precision would be needed to show whether the gradient actually was constant or not. Such a decline in the distribution ratio is to be anticipated for active transport because as the amino acid levels rise, (a) the influx rate will eventually fail to increase proportionally as the carrier approaches saturation, whereas (b) the efflux rate should continue to increase directly with the level of free amino acids in the cell. Kinetic studies by Heinz show that this is actually the case.

Secondly, a constant gradient might result from a secondary limitation placed upon the inward transfer process by the osmotic pressure arising from the gradient established.

Although the distribution ratio decreases with increasing load, at lower levels it tends to remain more nearly constant than the gradient, and can serve for comparisons of the activity within a restricted range. The distribution ratio also has special interest in that it determines the work required to transfer one molecule of amino acid into the cells, given that we know the true concentration inside. The work required to maintain a given distribution will of course also depend upon the rate of leakage from the cell.

The most important aspect of the steady-state distribution is the tendency of the transfer process to become saturated with increasing load. The curves have the form of adsorption isotherms, and imply that the amino acid enters into combination with cell components of limited capacity, either temporarily during entrance into the cell, or more permanently.

*Initial rates of transfer.* A better analysis is obtained by separate measurements of the rates of influx and efflux. Such measurements have been made by Heinz (26), working in this laboratory, by the use of 1-C<sup>14</sup>-glycine. As illustrated in Fig. 3, the rate of uptake by the ascites carcinoma cell even at 28° C. is so fast as to introduce considerable technical difficulty. Half the saturation transfer of

glycine was complete in 3 minutes. In order to minimize loss of the accumulated radioactive glycine from the cells they were centrifuged rapidly at just above the freezing point. Fig. 4 shows the rate of uptake of glycine (measured isotopically) in the presence of various

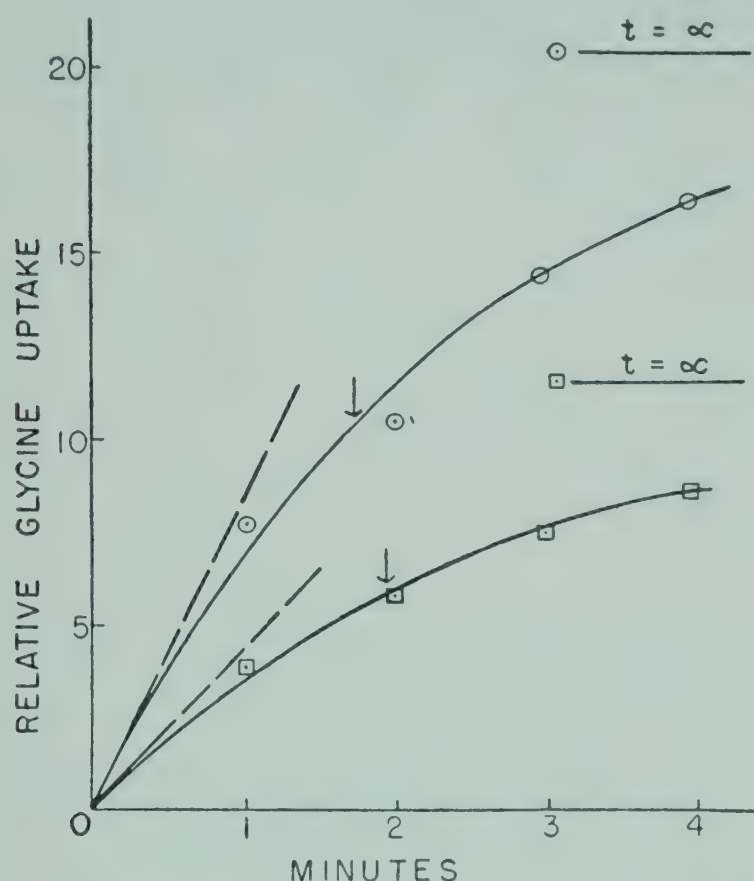


FIG. 3. Time-course of glycine transfer into ascites carcinoma cells (26). The encircled points show the uptake from a 0.5 millimolar glycine solution, the squares, from a 5 millimolar glycine solution, in each case at 28° C. and in millimoles taken up per 1 millimolar outside concentration per gram of dry cell weight. The horizontal lines at the right are the extrapolated values for infinite time, the dotted lines at the left the initial influx rates, both calculated by the equation  $U^x = U^x_{\infty}(1 - e^{-kt})$ . The arrows indicate the half-time for maximum uptake.

(From Heinz, E., *J. Biol. Chem.*, in press.)

levels of unlabeled glycine in the suspending fluid. The curves are drawn to fit the equation of Michaelis and Menten, as shown. A better test of fit is obtained in the upper line, where the equation has been rearranged to yield a straight line.

The results show clearly that as the rate-limiting step the glycine becomes bound to an acceptor of limited capacity. They do not tell us whether this binding is a step in an active transport, but they exclude the possibility of accounting for the asymmetry of amino acid distribution by a Donnan equilibrium. As an exception lysine



accumulation by *Strep. faecalis* has been considered by Gale as a Donnan effect, although distribution ratios higher than 20 to 1 were found, and the uptake process was readily saturated (27).

The previous accumulation of unlabeled glycine did not slow the subsequent accumulation of labeled glycine (26). If the accumulated glycine remained in association with the acceptor to which it

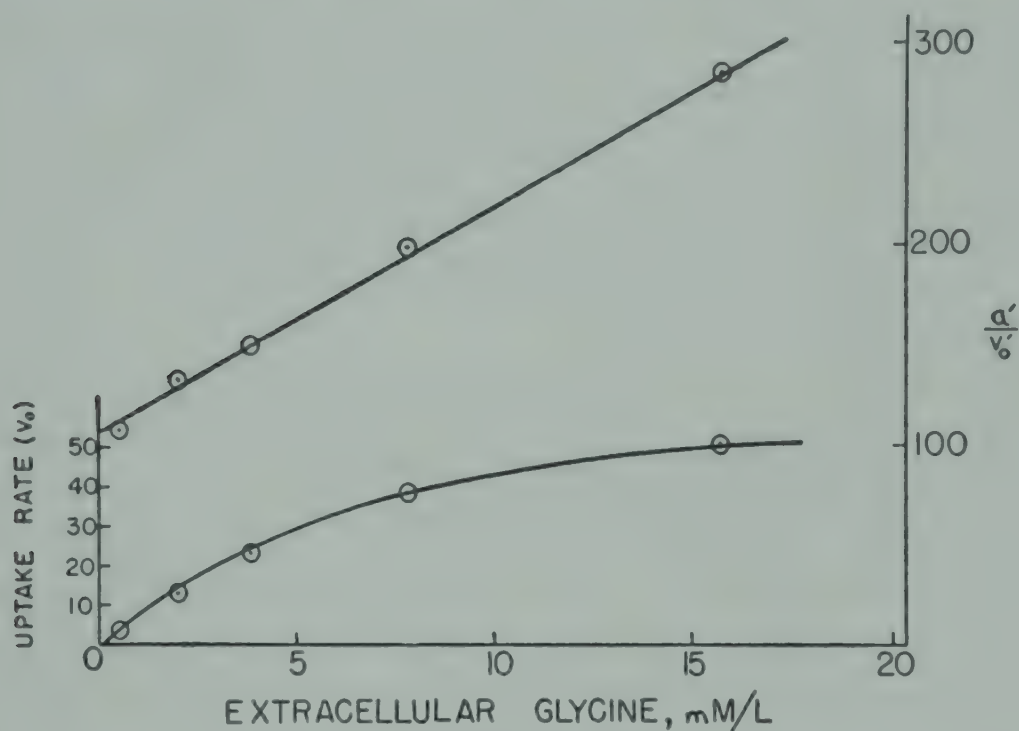


FIG. 4. Glycine influx rate as a function of the extracellular glycine level (26). The lower curve and the scale at the left shows the influx rate ( $V_o$ ) in micromoles of glycine per g. dry cell weight in two minutes at 29° C., measured isotopically. The curve is drawn from the equation of Michaelis and Menten in the form

$$V_o = K_v C_o \frac{a}{a + K_m},$$

where  $a$  = outside concentration,  $C_o$  = concentration of the hypothetical binding group, and  $K_v$  = velocity constant for the transport of the complex. The upper curve refers to the rearranged equation

$$\frac{a'}{V_o} = \frac{a + K_m}{K_v C_o},$$

where  $V_o$  and  $a'$  were measured in terms of the number of radioactive counts rather than the total amount of free glycine. The line shows the theoretical relationship.

(From Heinz, E., *J. Biol. Chem.*, in press.)

had been joined by the rate-limiting step, as shown in Fig. 5, one would expect that fewer places would be available for the subsequent uptake of the labeled glycine. The actual result indicates that the glycine in the cell does not remain in association with the same acceptor with which it is bound in the rate-limiting step.

Table 1 illustrates the finding that the initial rate of *efflux* is proportional to the apparent cellular level of previously accumulated

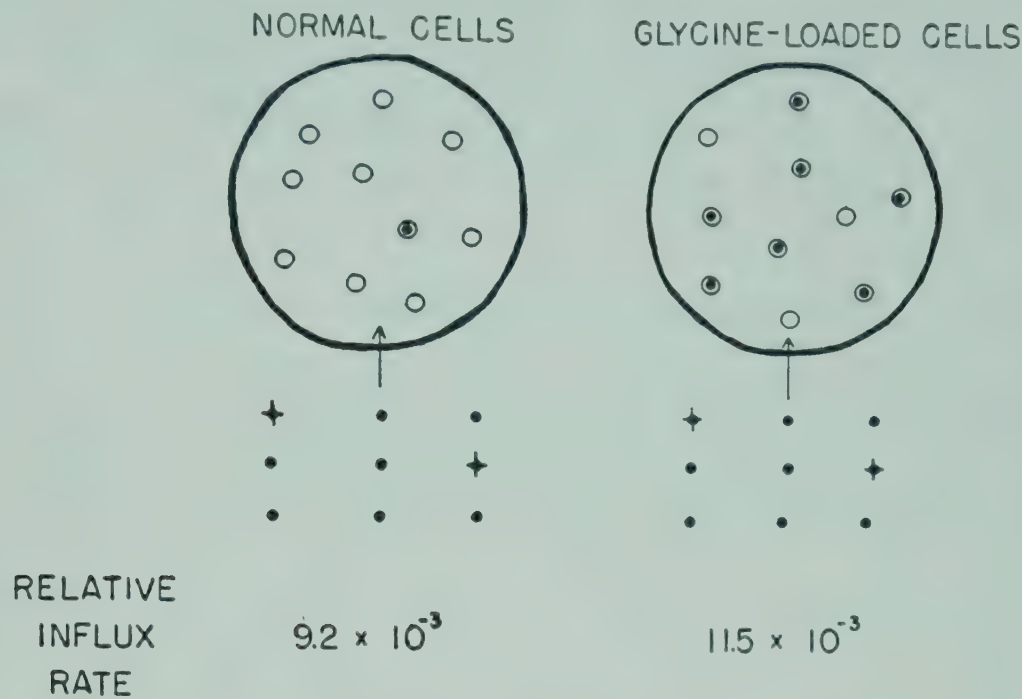


FIG. 5. Failure of the transfer process to be slowed by prior presence of glycine in the cell (26). ● Unlabeled glycine; + Labeled glycine; ⊙ Binding group bearing an unlabeled glycine. The influx rate is expressed in millimoles of glycine per 1 millimole/l. outside concentration per gram dry cell weight. The diagrams represent hypothetical cells containing a limited number of binding groups, upon the tentative theory that the accumulated glycine is in a bound form. In the cell on the right many of the binding positions have been filled by prior incubation with unlabeled glycine. The analytical results show that upon subsequent incubation with 1.4 millimolar labeled glycine the preloaded cells took up labeled glycine not slower (as would be expected) but faster than the normal. Therefore the above schematic representation is not correct.

TABLE 1

RELATION OF THE EFFLUX RATE FOR GLYCINE TO THE GLYCINE CONTENT OF CELLS

After the cells had taken up various amounts of labeled glycine, they were transferred to a fresh medium 20 millimolar in unlabeled glycine, and the efflux during 2 minutes was observed. The efflux is given in millimoles of glycine lost per millimole of glycine in a kilogram of cell water per gram of cell solids per minute.

After the experiment the cells were dried in the frozen state, the water restored, and particulates centrifuged down at 15,000 G. The table shows the distribution of labeled glycine between the supernatant phase (deproteinized by alcohol) and the particulate phase. (Data of E. Heinz, 26).

Total Cell Glycine mM./kg. water	Relative Efflux Rate	Radioactivity of Cells		Ratio
		Particulate phase c.p.m./g. H <sub>2</sub> O	Supernatant phase c.p.m./g. H <sub>2</sub> O	
1.0	$0.61 \times 10^{-3}$	28400	33100	1.16
6.0	$0.59 \times 10^{-3}$	18600	21700	1.16



glycine (26). This finding agrees with the supposition of a free state for most of the glycine in the cells and of diffusion as the rate-limiting step in its exit. The result cannot, however, be taken as proof of both propositions at once, since the second conclusion depends upon the first. Accordingly, independent evidence for the free state of the amino acids will be discussed below (section V).

#### IV. TEMPERATURE DEPENDENCE

In all cases studied the effect of temperature is not explicable on the basis of an adsorption phenomenon, but indicates that a chemical reaction is involved in the transfer process. Gale and his

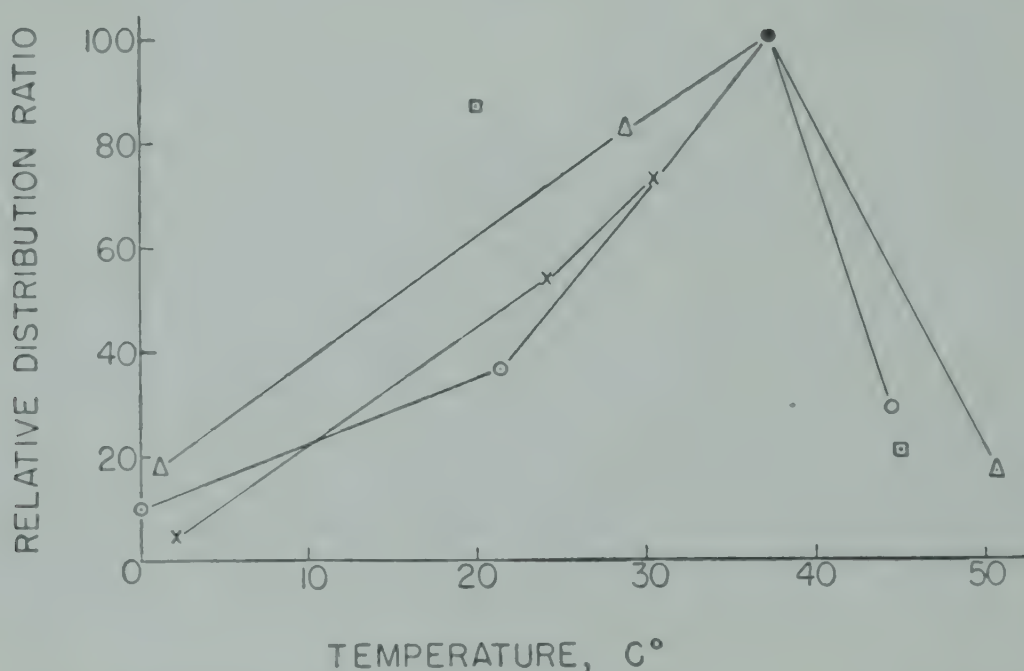


FIG. 6. Effect of temperature on the distribution of amino acids between the ascites carcinoma cells and the extracellular phase. The distribution ratio attained at 37.5° C. has been taken as 100. Δ, Glycine, Experiment 23; □, Glycine, Experiment 24; ○, L-α,γ-diaminobutyric acid; ×, L-tryptophan. (From ref. 18 and 41.)

associates showed that no accumulation of glutamate took place at 2° C. in his bacterial strains, and that the  $Q_{10}$  was above 2. The uptake of lysine by *Strep. faecalis* was exceptional in that the  $Q_{10}$  was only 1.4 and accumulation still occurred at 2° C. With the rat diaphragm the release of accumulated glycine upon cooling was demonstrated, with reconcentration upon warming (6). The exit of glycine, in contrast, was half as fast at 0° C. as at 36° C., giving a  $Q_{10}$  of about 1.2. Therefore this probably is merely diffusion.

Fig. 6 shows the effect of temperature upon the distribution ratios

that were reached by the uptake of various amino acids by avian erythrocytes and Ehrlich carcinoma cells. A high degree of temperature dependence is evident. Since accumulated amino acids are lost upon cooling, in order to argue that the amino acids are bound it is necessary to suppose that the bound form is broken by merely cooling the cells.

The effect of temperature upon the *initial rate* of glycine concentration by the neoplastic cells was investigated by Heinz (26) by the addition of 1-C<sup>14</sup>-glycine to the extracellular phase. Although glycine reaches only a moderately lower steady-state distribution ratio at 28° C. compared with 38° C. (Fig. 6) a  $Q_{10}$  of 1.8 was found for the initial rate of influx in the range of 24° to 32° C., a coefficient indicating again that a chemical reaction is involved in the transfer.

## V. EVIDENCE BEARING UPON THE STATE OF "FREE" AMINO ACIDS IN CELLS

### A. State of amino acids in broken cell preparations.

Table 2 shows the distribution of glycine between lysed erythrocytes confined in a cellophane bag, and 0.9% sodium chloride (17).

TABLE 2

#### FAILURE OF LYSED ERYTHROCYTES TO ACCUMULATE AMINO ACIDS

The lysates, prepared by freezing and thawing, were enclosed in a cellophane bag and dialyzed 22 hours at 24° C. against an equal volume of 0.9% NaCl. The results are expressed in mM per kilo of water. (From Christensen, Riggs, and Ray, *J. Biol. Chem.*, 194, 41, 1952).

Origin	Amino Acid Determined	Concentration found	
		Lysate phase	Outside bag
Human	Glycine	0.48	0.49
	Alanine	1.17	1.16
Duck	Glycine	1.45	1.40
	Alanine	0.98	1.02

The cells were broken by freezing and thawing. The concentrations are expressed in millimoles per kg. of water. No binding by macromolecular components of the hemolysates is evident.



Table 3 shows the distribution of glycine and of amino acids in general between lysed Ehrlich carcinoma cells and Krebs' Ringer-bicarbonate medium (28). The cells were broken by exposure to nitrogen pressures of 200 pounds per square inch, followed by sudden release of the pressure. A minimal amount of "free" amino acid appears to have been "accumulated" in the lysate. The pres-

TABLE 3

DISTRIBUTION OF AMINO ACIDS BETWEEN LYSATE OF CARCINOMA CELLS (BY RELEASE OF HIGH NITROGEN PRESSURE) AND KREBS' RINGER-BICARBONATE ACROSS A CELLOPHANE MEMBRANE

No intact cells could be detected. ATP added to 1.3 mM/l. The amino acids present originated from the cells. Dialysis 5 hours. The concentrations are in millimoles per kg. water. (Christensen, Riggs, and Fischer, unpub.)

Amino Acid Determined	Concentration		Ratio
	Lysate phase	Outside phase	
All $\alpha$ -amino acids *	12.0	9.24	1.3
Glycine	1.87	1.52	1.23

\* Manometric ninhydrin method.

ence of a small bound portion might be attributable to a "carrier" still present in the broken cells, or alternatively the small extra amount of amino acid in the lysate phase may have been due to incomplete equilibrium, since the free amino acids present originated from this phase, partially by a continuing proteolysis.

Table 1 shows that labeled glycine does not become associated to a significant extent with the particulates obtained after removing the water from the carcinoma cells in the frozen state (Heinz, 26). The evaporated water was in this case restored, and after incubation the particulates were centrifuged down for analysis.

Preliminary results indicate that the mitochondria of rat kidney may contain up to twice as high a concentration of apparently free glycine (i. e., material yielding formaldehyde with ninhydrin) per unit of water as does the medium in which they are incubated (29). Preliminary experiments, however, have failed to demonstrate accumulation. The study is complicated by the continuous generation of glycine by the mitochondria. The distributional asymmetry observed



is of a similar magnitude to that demonstrated by MacFarlane and Spencer (30) and by Stanbury and Mudge (31) for potassium with mitochondria. Perhaps this observation continues the analogy between potassium and amino acid distribution.

The failure of broken cells to show any substantial degree of accumulation of amino acid was perhaps to be expected from the following consideration: the process of accumulation is already blocked if potassium is supplied outside the cell at a concentration half as great as that inside the cell (see below). The asymmetry of potassium distribution is an essential feature of the cellular environment and cannot readily be reproduced with broken cells. If accumulation is produced by active transport, cell breakage will make the process ineffective by joining together the separate compartments involved. But if accumulation occurs merely by the binding of the amino acids to cell components, cell breakage might also interrupt the process if the new conditions were not favorable to that binding. Therefore evidence obtained on whole cells as to the internal state of the amino acids of the cell appears to be more convincing.

### *B. State of the Amino Acid in Intact Cells.*

#### *1. Indications from the quantities of amino acids which can be accumulated.*

If one attributes to a bound form the *extra* amino acids (above the extracellular level) which can be found in cells, he must look for a binding agent present at a 60 millimolar (for glycine) to 140 millimolar concentration (for  $\alpha$ ,  $\gamma$ -diaminobutyric acid). If he assigns all the amino acids that can be transferred into the cell to a bound form, the requirement becomes much larger still. One can at once exclude sulfur compounds, phosphorus compounds, and many other classes of compounds on the basis of this requirement. Assigning arbitrarily an equivalent weight of 120 to the hypothetical binding agent, it would need to represent about 10 per cent of the cell solids, and accordingly it should not readily escape detection.



## 2. *Transfer of water with the amino acid.*

The best evidence for the free state of amino acids in cells comes from the water shifts which accompany the uptake of amino acids by the ascites tumor cells. These cells respond in an approximately osmometric manner by losing and gaining water according to the tonicity of the extracellular phase and in association with migrations of inorganic ions. Fig. 7 shows the correspondence between the

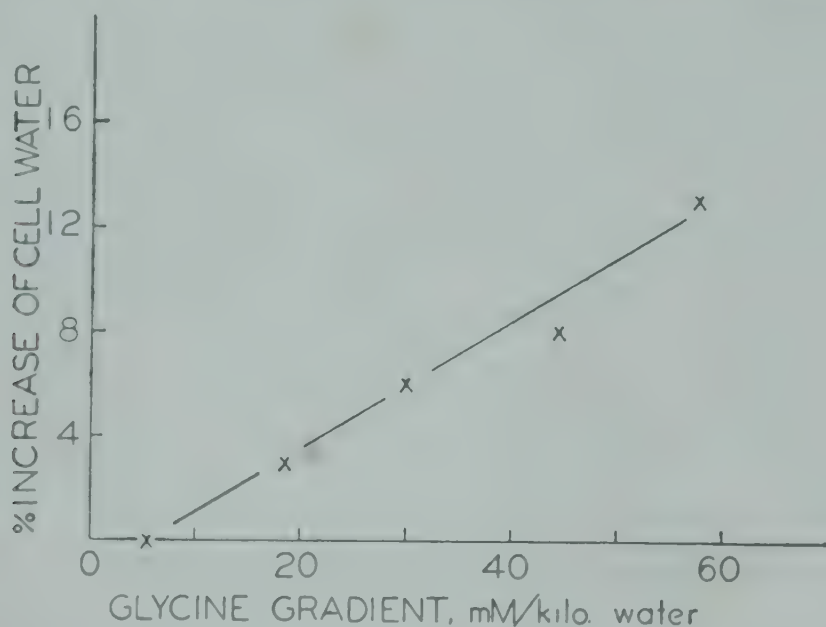


FIG. 7. Water uptake by carcinoma cells associated with the development of a glycine gradient. The line corresponds to the uptake of the extra glycine approximately as a 0.7 isotonic solution. (Riggs, Mothon, and Christensen, unpub.).

glycine gradient established and the water transferred. The glycine has apparently entered the cells as a 0.7 isotonic solution. A precise determination by this method of the percentage of glycine which is free in the cells is difficult because small shifts of other solutes may be overlooked. The migration of inorganic ions into the cells in this experiment was excluded as an important factor in the water shift.

An exaggerated illustration of this behavior was obtained by performing the experiment in the presence of 20 millimolar pyridoxal (32). Under these circumstances cell swelling was very greatly increased by the concomitant presence of increasing amounts of glycine in the medium. These increases correspond, however, with the uptake of glycine as an approximately 0.3 M solution.

Water transfer is also much greater during the accumulation of

L- $\alpha$ , $\gamma$ -diaminobutyric acid, because much larger amounts of this amino acid are transferred into the cell. Here again the water transfer is closely related to the amino acid transfer when the simultaneous displacement of potassium (Fig. 8) is taken into account. Furthermore, the swelling can be controlled by starting with a hypertonic salt solution; in this case higher levels (up to 160 millimolar) of the amino acid are reached in the cell. One must conclude that most of an amino acid upon introduction into the cell, either (a) is released to the free state, or (b) becomes combined, molecule

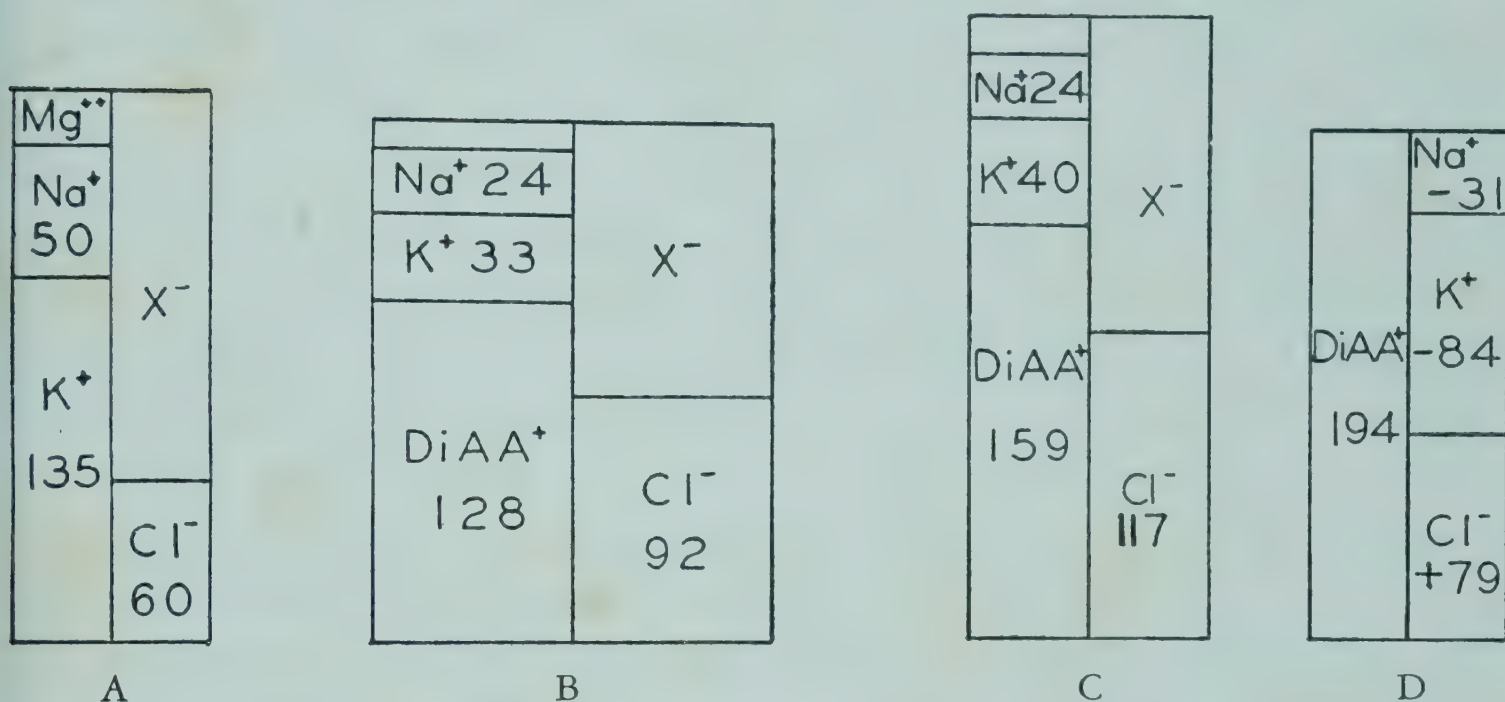


FIG. 8. Uptake diaminobutyrate (DiAA<sup>+</sup>) by the ascites carcinoma cell. A, typical normal concentrations of ions in the cell water in milliequivalents per kg. B, after two hours in isotonic Krebs' Ringer-bicarbonate containing originally 30 millimolar DiAA<sup>+</sup>Cl<sup>-</sup>. C, after 3 hours in a medium containing 40 millimolar DiAA<sup>+</sup>Cl<sup>-</sup> and made 1.5 times isotonic by sodium chloride addition. D, ions transferred into cells in C, in milliequivalents per kilo. of original cell water. The uptake of water is indicated by the thickening of bars B and C. (From Christensen, Riggs, Fischer, and Palatine, *J. Biol. Chem.*, 198, 15, 1952).

for molecule, with a substance formed specifically (or retained specifically) in response to the entrance of the amino acid, or (c) becomes combined to a cell component, at the same time releasing, molecule for molecule, another substance. Neither possibility (b) nor (c) seems very likely, especially because of the very large amounts of the hypothetical substances required. No simple binding of the amino acid, whether to a univalent or polyvalent acceptor, would explain the water transfer.



The foregoing considerations cannot be applied directly to the bacterial cells studied by Gale (8), because these fail to swell during amino acid accumulation.

3. *Preservation of net charges of amino acids in cells.* The amino acids retain not only their osmotic activity but also their ordinary state of net charge in the cells. If they were accumulated in a bound state, the binding might abolish either a negative or positive charge, but this is clearly not the case, as the following results show:

(a) The uptake of large amounts of neutral amino acid is accompanied only by approximately reciprocal shifts of sodium and potassium (20).

(b) Along with glutamate, potassium and sodium ions are taken up, as required for electroneutrality. This effect has been observed in cerebral cortex slices (16), in the ascites tumor cells (20), and in bacteria (33). In the carcinoma cells in Krebs' Ringer-bicarbonate medium the two cations share about equally in the uptake. One cannot draw up a balance sheet for these shifts unless the various anions (e. g.,  $\alpha$ -ketoglutarate) arising from glutamate are also measured. Whether the accumulation of potassium and sodium by mitochondria from  $\alpha$ -ketoglutarate or glutamate solutions is occasioned primarily by the accumulation of the anion or the cations, or both, has not been established, although the finding by Stanbury and Mudge (31) that the exchange of mitochondrial potassium was stimulated by the presence of oxygen appears to speak for a specific accumulation of potassium.

(c) The uptake of very large amounts of the organic cation, L- $\alpha,\gamma$ -diaminobutyrate (34), is accompanied by shifts of chloride and of potassium very near to what is required for electroneutrality (Fig. 8).

## VI. STIMULATION BY PYRIDOXAL

Figs. 9 and 10 show the effects of pyridoxal added to the suspending medium on the accumulation of glycine by the Ehrlich carcinoma cells, working at two different glycine levels (35, 32). The upward extension of the optimum pyridoxal level when the

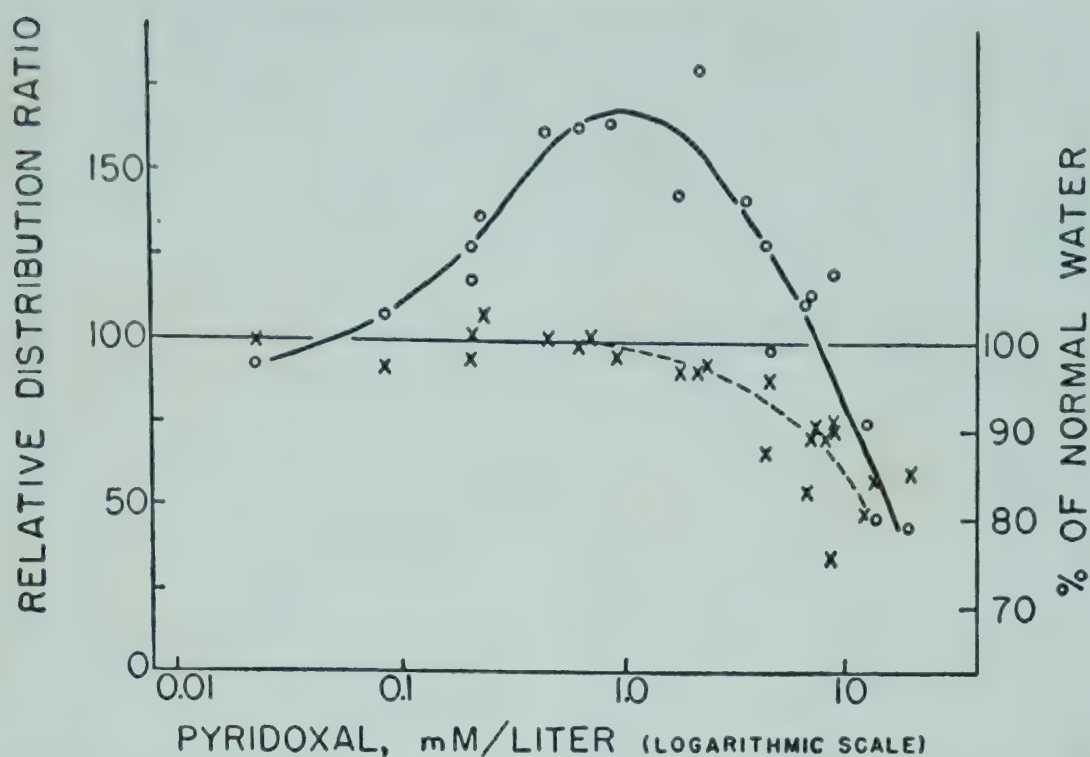


FIG. 9. Action of pyridoxal on the concentration of glycine from a 2 mM solution by ascites carcinoma cells. O, distribution ratio, glycine of cells to glycine of suspending fluid, relative to that attained in the absence of added pyridoxal (scale at right). X, water content of incubated cells compared with normal (scale at right). (From Christensen, Riggs, and Coyne, *J. Biol. Chem.*, 209, 413, 1954).

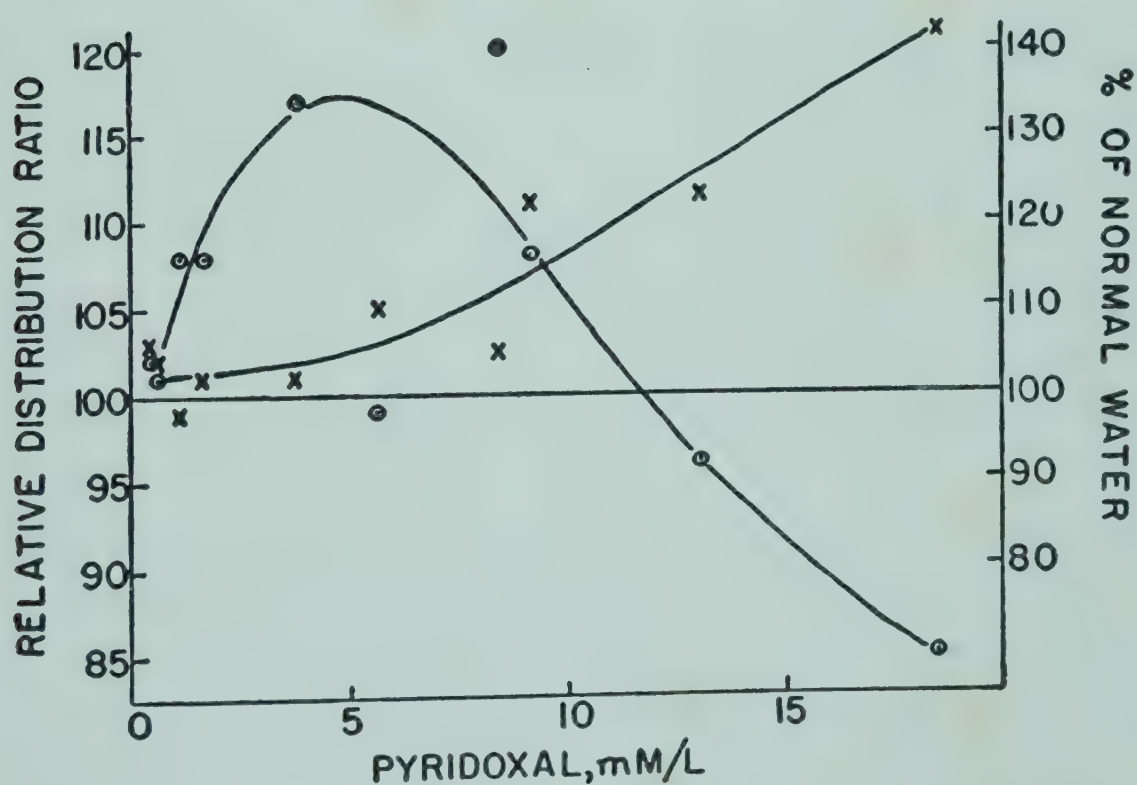


FIG. 10. Action of pyridoxal on the concentration of glycine from a 25 mM solution. Mode of designation same as in fig. 9. (From Christensen, Riggs, and Coyne, *J. Biol. Chem.*, 209, 413, 1954).



glycine level is increased is probably attributable to an overbalancing on the one hand of the cell shrinkage produced by pyridoxal at higher levels, by the swelling which glycine transfer occasions, on the other hand. The accumulation of tryptophan, diaminobutyrate, and methionine was also stimulated by pyridoxal.

There are other chemical agents (see section IX) which stimulate the concentration of glycine, although not at levels as low as 0.5 millimolar. The more significant finding, shown in Table 4, is that the accumulation of glycine by ascites cells grown in B<sub>6</sub>-deficient mice was distinctly subnormal. Furthermore, these cells were stimulated in their glycine uptake by levels of pyridoxal which were not effective in the normal carcinoma cells. A normal participation of pyridoxal in amino acid transfer into cells seems to be indicated. The extra glycine taken up often exceeded the total amount of pyridoxal present, so that a mole-for-mole binding of glycine with pyridoxal is not a possible explanation.

TABLE 4

DEPRESSED CONCENTRATIVE ACTIVITY OF TUMOR CELLS FROM PYRIDOXINE-DEFICIENT MICE

Two hours of incubation in Krebs' Ringer-bicarbonate medium, 27 millimolar in glycine. (From Christensen, Riggs, and Coyne, *J. Biol. Chem.*, 209, 413, 1954).

	Trials	Cellular glycine	
		Extracellular glycine	Glycine gradient
Normal cells	7	$2.98 \pm 0.06$	$42.3 \pm 1.9$
Pyridoxine-deficient cells	4	$2.29 \pm 0.09$	$33.5 \pm 2.6$
Same, + 0.4–0.8 mM pyridoxal	3	$2.68 \pm 0.18$	$38.2 \pm 3.1$

One might suppose that the "normal" carcinoma cell is pyridoxine-deficient, since it is so readily stimulated in this function by added pyridoxal. Even a previous incubation of the cells with pyridoxal, however, did not bring them to a full activity; these cells could still be further stimulated when pyridoxal was added along with the glycine. Therefore the simultaneous presence of pyridoxal and the amino acid appears to be advantageous to maximum glycine concentration. This behavior is what might be expected of the



carrier or a precursor of the carrier, if one considers that the carrier needs to be kept at higher concentration at the outer limit of the cell boundary than within the osmotic barrier (36).

Pyridoxal phosphate is not more, but probably less, effective than pyridoxal. Pyridoxamine and pyridoxine are ineffective, whereas desoxypyridoxine inhibits glycine concentration (32).

Snell has outlined the changes in the stability of various structures of the amino acid molecule which can occur upon combination with pyridoxal (37). The suggestion made here is that combination with pyridoxal or a derivative of pyridoxal might serve in the transfer of an amino acid from one point to another, with further modification of the amino acid molecule occurring only as the presence of specific enzymes permits. The action of pyridoxal could of course pertain to subsidiary reactions rather than to the transfer reaction itself; or conceivably a pyridoxal derivative somewhat different from that involved in transamination may be involved in the transfer process.

*4-Nitrosalicylaldehyde.* Many of the non-enzymatic catalytic effects of pyridoxal were also obtained with its benzene analog, 4-nitrosalicylaldehyde, by Ikawa and Snell (38). The strong electron-attracting nitro group serves instead of the pyridine ring-nitrogen. 4-Nitrosalicylaldehyde catalyzes the dehydration of serine, the desulfhydration of cysteine, the cleavage of threonine to glycine, and the deamination of glutamic acid. Accordingly a sample of this aldehyde, the gift of Dr. Snell, was tested very recently for its effect on glycine accumulation. Preliminary results are shown in Fig. 11. Here again is seen the same remarkable association between the stimulation of amino acid transfer and the shrinkage of the cells (presumably here also due to potassium exodus), and between the inhibition of amino acid transfer and the swelling of the cells. The concentrations required in the suspending fluid are somewhat less than for pyridoxal.

The preliminary indications that this abnormal analog serves at least as well as pyridoxal has some strong implications. First, the likelihood that the 4-nitrosalicylaldehyde must be modified



enzymatically before it can serve is less than for pyridoxal; for one thing, it does not have an aliphatic hydroxyl group which may be phosphorylated. Secondly, it seems less likely that 4-nitro-salicylaldehyde facilitates cellular energy-yielding reactions rather than the transfer process itself. Finally, one may be less inclined to assign to pyridoxal a coenzyme function here, knowing that an artificial analog also serves.

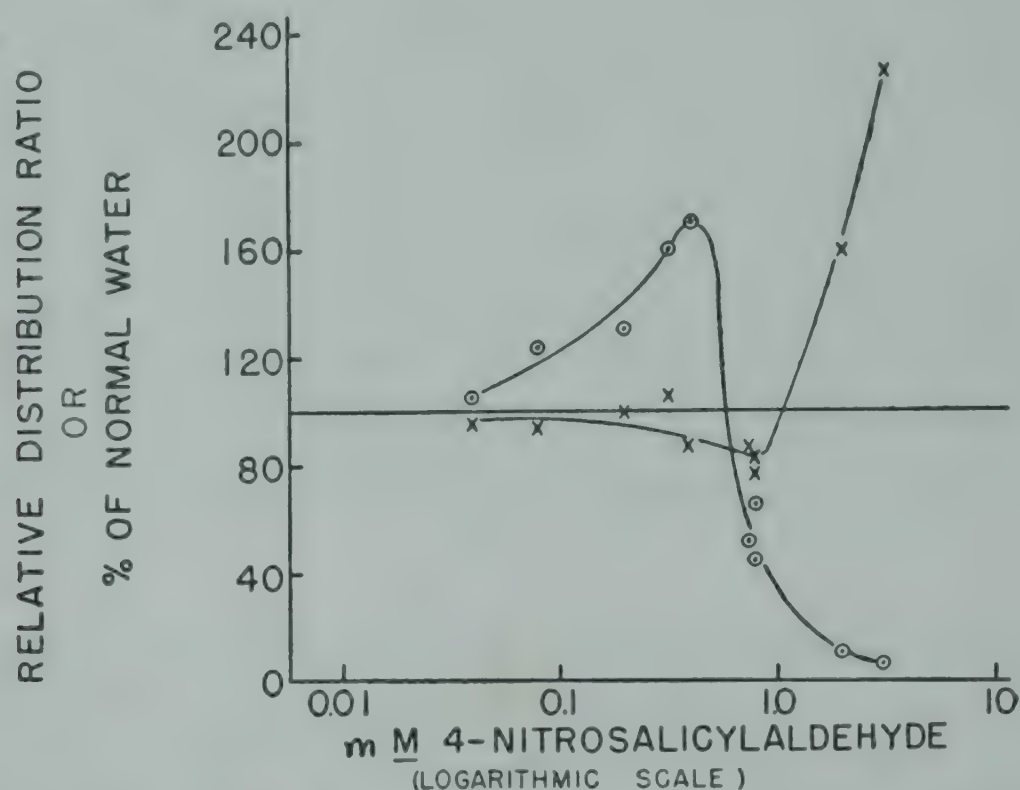


FIG. 11. Effect of 4-nitrosalicylaldehyde on the distribution of glycine and of water, between ascites tumor cells and the suspending fluid.  $\odot$ , Distribution ratio, cellular glycine/extracellular glycine, setting the ratio in the absence of the aldehyde equal to 100.  $\times$ , per cent of normal water content of the cells. (Riggs, Mothon, and Christensen, unpub.).

## VII. INHIBITORS OF AMINO ACID CONCENTRATION

Table 5 summarizes the action of a number of inhibitors upon amino acid accumulation. Inhibitors of respiratory metabolism or associated phosphorylation interfere much more seriously for some cells than for others, and scarcely at all for erythrocytes. The implication is that the interference is only with the energy source and not with the transfer process itself, or else that the transfer process is not the same for these various cells. Erythrocytes are practically unaffected in their activity by the exclusion of oxygen, even where carbon monoxide was used to displace oxygen.

TABLE 5

ILLUSTRATIONS OF INHIBITORY EFFECTS ON THE CONCENTRATION OF GLYCINE  
BY ASCITES CARCINOMA CELLS

Glycine, 2 millimolar. The distribution ratio in the absence of the inhibitory factor was taken as 100. (From ref. 19, 23, 40 and 43).

Inhibitor	mM./l.	Relative Distribution Ratio Glycine
Cyanide	5	16
2,4-Dinitrophenol	1	30
Absence of oxygen	—	26
Arsenate	10	42
Iodoacetate	5	18
Fluoride	10	30
Malonate	40	29
Aureomycin	2	47
Chloromycetin	5	39
Clupeine	(0.1%)	44
L-alanine	11	24
L-leucine	11	55
L-tryptophan	11	104
$\beta$ -Chloro-L-alanine	2	31
Sodium ethylenediamine tetraacetate (Versene)	1	55

Because pyridoxal derivatives appear to take part in amino acid transfer and because copper, aluminum, or iron participates in the non-enzymatic catalytic activities of pyridoxal (39), interest was considerably heightened in the possible participation of trace metals in the transfer process. There is also the curious association in Wilson's disease between deranged copper metabolism and disturbed amino acid distribution. In *Staph. aureus* (40) the accumulation of glutamate is activated by either manganese or magnesium, and 8-hydroxyquinoline inactivates the process. Calcium was an essential component of the medium from which glycine accumulation was produced by the rat diaphragm (6), but it has been without effect for erythrocytes and the Ehrlich carcinoma cell. 8-Hydroxyquinoline has little effect, whereas ethylenediaminetetraacetic acid (41) is a moderately active competitive inhibitor of amino acid transfer (Table 5). Its effect can be reversed by several metals which would be expected to bind it. There are no clear indications as to whether



this chelating agent and the amino acids are competing for a metallic or a non-metallic carrier. Metzler, Ikawa, and Snell (37) commented on the paucity of evidence for metal participation in enzymatic reactions in which pyridoxal is involved, and suggested the possibility that a portion of the enzyme protein could fill the role played by metals in the non-enzymatic reactions.

The concentration by the ascites tumor cell of any of the neutral amino acids is competitively inhibited by the presence of one or more of the other neutral amino acids, but not by the anionic or cationic amino acids; in fact, the latter have shown themselves to be generally somewhat stimulatory (23). Avoidance of inhibitory effects of other amino acids probably explains the better concentration of several amino acids from Krebs' Ringer-bicarbonate medium than from the native ascitic plasma, containing as it does a mixture of amino acids.

The severity of the inhibitory action usually increases with the extent to which the inhibiting amino acid is itself concentrated, although tryptophan is an exception. Tryptophan uptake is also an exception in not stimulating water transfer; possibly a smaller fraction of it remains free in the cell. Another exception is  $\beta$ -chloro-L-alanine, which is not concentrated appreciably but is an unusually effective inhibitor; this amino acid, however, is dechlorinated in the presence of the tumor cells. The lack of inhibition by aspartic acid is not inconsistent, since it gave little indication of being accumulated. The question of the accumulation of glutamate will be discussed below.

The inhibitions among the neutral amino acids are interpreted as indicating a common step in their transfer. The existence of one transfer system of low specificity for a large number of amino acids is also supported by the finding that many synthetic amino acids of abnormal structure are accumulated. The absence of interference between neutral and anionic and between neutral and cationic amino acids might suggest that a different transfer system exists for each class, although this does not necessarily follow.

A somewhat different pattern of inhibitions and facilitations of glutamate transfer by various amino acids has been found in *Staph. aureus* (42). In association with these inhibitions, in some cases



peptides of glutamic acid were released into the medium. An activation of the amino group of glutamate was inferred; the possibility was considered that peptide bond formation, or transpeptidation, occurred during transfer into the cell. No counterpart to this event has been detectable in the carcinoma cells (23). Although the formation of a peptide bond is a possibility for the binding of an amino acid during its transfer into the cell, the accumulation of an amino acid such as  $\alpha$ -aminoisobutyric acid (23) through a peptide intermediate appears rather unlikely.

### VIII. WHAT AMINO ACIDS ARE CONCENTRATED?

Very few amino acid structures have been encountered which are not concentrated by the Ehrlich ascites carcinoma cell. Several effects of structure are evident from comparisons:

1. Although L-amino acids are usually the more strongly concentrated, the D-isomers are also accumulated by the ascites carcinoma cell. D-Amino acids compete with L-acids for transfer (23). A carrier which will combine with either isomer is visualized, with the advantage of the L-form still to be explained.

2. The degree of concentration falls off rapidly with an increasing number of carbons in an aliphatic hydrocarbon side-chain (23). Illustrative distribution ratios obtained under similar conditions are: glycine 7, alanine 4, valine 1.4, isoleucine 1.5, leucine 1.13. The difference between alanine and glycine is much larger if the comparison is made at a 30 or 50 millimolar level; that is, for alanine the transfer apparatus is much more easily saturated.

3. The degree of concentration is increased by the presence of electron-attracting groups on the side-chain (23), as indicated by the following comparisons of distribution ratios made either simultaneously or under similar conditions:

L-proline 2.5; L-hydroxyproline 3.1.

L-valine 1.4; L-ornithine 3.9; L-histidine 4.1; L-methionine 4.7; L-citrulline 5.1; L- $\alpha,\gamma$ -diaminobutyric acid 20.6.

L-leucine 1.13; L-isoleucine 1.5; L-phenylalanine 2.6; 2-pyridyl-DL-alanine 6.9.



The results appear to indicate that, other things being equal, the degree of concentration rises as the  $pK_a$  of the carboxyl and amino groups falls. This would suggest that the chemical reactions involved in the transfer are favored either by the presence of the carboxyl group in the *charged* form or by the presence of the amino group in the *uncharged* form. The formation of acyl derivatives, Schiff's bases, and the like with the amino group would be favored by the presence of a higher proportion of the uncharged amino form. Contrariwise, the formation of thioester or amide links to the carboxyl group should occur *less* readily with the charged carboxylate group. Other considerations (23) have pointed to the amino group as the more likely site of the reaction.

The relation of pH to the degree of concentration is pertinent to this question. The optima for glycine and histidine lie between 7.3 and 7.9 (23); the very rapid decrease above pH 8 is accompanied by considerable disintegration of the cells, whereas the cells do not change noticeably in appearance during the decline between pH 7 and 6.5. The indications are that the form of histidine which has no net charge, rather than the cationic form, is the one which is concentrated. Gale has noted that lysine accumulation by *Strep. faecalis* increases up to pH 9.4, its isoelectric point (43).

4. The introduction of a methyl group on the  $\alpha$ -carbon increases the degree of accumulation (44). For example,  $\alpha$ -aminoisobutyric acid was concentrated 4.8 times, DL-isovaline 5.1 times, whereas L-valine was only slightly concentrated under similar conditions.

Table 6 shows that  $\alpha$ -methyl-DL-methionine is concentrated more than either ethionine or methionine, and that exceptionally large gradients can also be established with  $\alpha$ -methyl-DL-serine, DL-isovaline,  $\alpha$ -aminoisobutyric acid and  $\alpha$ -methyl-DL-asparagine. These findings indicate that the concentration reactions do not involve the removal of an alpha hydrogen atom. One explanation for the stronger accumulation is possible if a Schiff's base with a pyridoxal derivative is formed during the transfer process. The absence of the  $\alpha$ -hydrogen would prevent the electromeric shifts of the double bond which characterize the pyridoxal derivatives (37), for example, formulation



TABLE 6  
CONCENTRATION OF  $\alpha$ -METHYL-AMINO ACIDS BY CARCINOMA CELLS  
(Riggs, Mothon, and Christensen, unpub.).

	Cellular mM./kg. H <sub>2</sub> O	Extra- cellular mM./kg. H <sub>2</sub> O	Distribution ratio	Gradient mM./kg. H <sub>2</sub> O
1. $\alpha$ -Methyl-DL-methionine *	61.1	18.5	3.30	43
DL-Ethionine	54.4	18.0	3.02	36
DL-Methionine	54.9	19.1	2.87	36
2. $\alpha$ -Methyl-DL-serine *	73.9	18.1	4.08	56
$\alpha$ -Aminoisobutyric acid	52.2	17.0	3.07	35
$\alpha$ -Methyl-DL- $\alpha$ -aminobutyric	71.9	17.7	4.06	54
3. $\alpha$ -Methyl-DL-aspartate *	9.76	22.4	0.44	-13
$\alpha$ -Methyl-DL-asparagine *	53.6	13.6	3.94	40
$\alpha$ -Methyl-DL-glutamate *	7.61	20.6	0.37	-13

\* Kindly provided by Dr. Karl Pfister, Merck and Company.

X (see Fig. 12). Therefore the  $\alpha$ -methyl group might prevent the diversion of portions of the hypothetical carrier-amino acid complex into forms which could not redissociate directly.

5. The presence of a second carboxyl group may interfere with accumulation.

Whereas  $\alpha$ -methylasparagine is concentrated well,  $\alpha$ -methylaspartic and  $\alpha$ -methylglutamic acids are not accumulated at all. For the aspartic acid derivative this is not strange, since aspartate itself appears not to be concentrated by these cells (23). We are led to the suspicion that the transfer of glutamate as such into the cells is also poor, and that the observed transfer involves a removal of the alpha hydrogen, perhaps a transamination; the latter appears to be the case for its transfer through the intestinal mucosa (45). It may be recalled that in the experiments of Stern et al. (16) on the accumulation of glutamate by slices of cerebral cortex, transaminase activity was abundant. Incidentally, in most experiments on glutamate distribution the analyses have also included glutamine. If glutamate transfer were limited to a restricted permeation as such



plus a transfer in a different form, e. g., a transfer of the amino group to  $\alpha$ -ketoglutarate and other keto acids in the cell, the kinetics of transfer inward and outward might be greatly modified from those applying to glycine, for example. The results in any case emphasize a difference in the transfer of the dicarboxylic acids from that for neutral amino acids.

6. The introduction of a second amino group intensifies accumulation.

The homologous series, lysine, ornithine,  $\alpha,\gamma$ -diaminobutyric acid and  $\alpha,\beta$ -diaminopropionic acid are considerably more strongly concentrated than the corresponding monoamino acids studied, namely, the leucines, valine,  $\alpha$ -aminoisobutyric acid, and alanine. (Unfortunately,  $\alpha$ -amino-n-butyric acid has not been studied). What is more striking is the magnitude of the effect of shortening the chain in the diamino acid series. Although there is increased uptake with shorter chains among the monoamino acids, for the diamino acids the increase in the degree of accumulation is tremendous in going from ornithine to  $\alpha,\gamma$ -diaminobutyric acid;  $\alpha,\beta$ -diaminopropionic acid is concentrated almost as strongly as diaminobutyric. The affinity for diaminobutyric acid is so great as to be lethal; the cells accumulate the amino acid until almost all of their potassium is replaced by the organic cation, and until the cell is tremendously swollen by the uptake of amino acid, chloride, and water together (see Fig. 8). The movements of ions are electrically balanced, and the movement of water is such as to indicate that the amino acid is free within the cell. The liver cell of the intact animal exchanges about half of its potassium for the cationic amino acid, but here the uptake appears to be limited to this exchange process, presumably because of exclusion of the chloride ion.

The main problem is to explain the exceptional concentrative uptake of this amino acid, and to gain from it any implications as to how the transfer process operates. The reaction of this amino acid with a "carrier" might be favored by two circumstances:

(a) The spatial separation of the two amino groups in  $\alpha,\gamma$ -diaminobutyric acid (and in diaminopropionic acid) is such as to



permit the formation of stable rings of 5 or 6 members with an atom of the "carrier." The abruptness of the change in extent of transfer in going from ornithine to  $\alpha,\gamma$ -diaminobutyric acid appears to be best explained on such steric grounds. Extending the views of Snell et al. (37), one might speculate on the formation of a derivative with pyridoxal of the character of formulation A (Fig. 12) that might be more favorable to transfer than formulation VII. The studies of Albert (46) show that the copper salts of diaminopropionic and diaminobutyric acid involve mostly the two amino groups (corresponding to formulation A) rather than the  $\alpha$ -amino group and the carboxyl group.

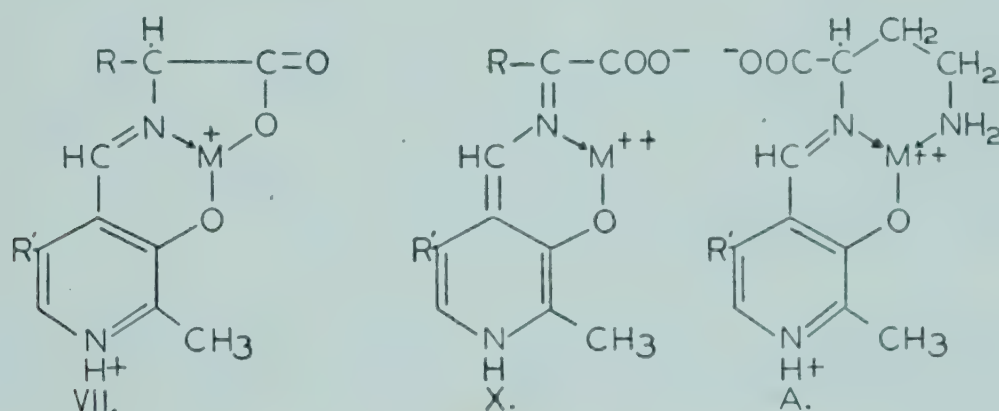


FIG. 12. Hypothetical formulations of pyridoxal derivatives of amino acids. VII and X are drawn from the structures of the same number of Metzler, Ikawa, and Snell (37). The presence of a methyl group on the  $\alpha$ -carbon should prevent electromeric shifts to formulation X, and all other formulations of Snell (37) VIII to XIII and XIV and XV. The presence of an amino group on the  $\beta$ - or  $\gamma$ -carbon might permit the formation of structures like A in competition with the formation of VII.

(b) The nearer the second amino group lies to the  $\alpha$ -amino group, the lower the  $pK_a$  of the alpha amino group. Values of 8.24 for diaminobutyrate and 6.69 for diaminopropionate were obtained by Albert (46). The stimulating effect of structural features which lower the basicity of this group has already been mentioned; derivatives of the type shown in Fig. 12 would be more stable at pH 7 even if the second amino group is not involved in the complex. To defend this explanation one might need to argue that an optimum degree of stability was exceeded in going from diaminobutyric acid to diaminopropionic acid. A redissociation is of course also necessary in the carrier function.



An additional illustration of the stimulation by a nearby nitrogenous group and of the importance of the spacing between the two groups is provided by a series of pyridyl amino acids (Table 7).

TABLE 7

STRUCTURAL EFFECTS AMONG PYRIDYL AMINO ACIDS ON ACCUMULATION BY CARCINOMA CELLS

Ten millimoles per liter of each amino acid added to cell suspension. Concentrations are in millimoles per kilogram of water. (From Riggs, Christensen, and Coyne, *J. Biol. Chem.*, 209, 395, 1954).

Substance	Concentration		
	Cells	Fluid	Ratio
2-Pyridyl-DL-alanine *	39.7	5.78	6.87
3-Pyridyl-DL-alanine *	30.8	9.04	3.41
$\gamma$ -(2-Pyridyl)-DL- $\alpha$ -aminobutyrate †	33.3	9.36	3.56

\* Gift of Dr. Karl Neimann, California Institute of Technology.

† Gift of Dr. J. H. Burckhalter, University of Kansas.

A separation by more than three carbons was disadvantageous, as illustrated by both 3-pyridylalanine and  $\gamma$ -(2-pyridyl)- $\alpha$ -aminobutyric acid compared with 2-pyridylalanine (41). Here the facilitation of uptake by the second nitrogenous group was obtained without the introduction of a positively charged group. The strong accumulation of compounds of this nature makes possible the colorimetric observation of amino acid uptake. With  $\gamma$ -(2,4-dinitroalanino)-L- $\alpha$ -aminobutyric acid an intense, even yellow coloration of the cells was obtained at a magnification of 900 times. This particular amino acid was not sufficiently soluble to be ideal for the purpose. With the highly soluble  $\gamma$ -amino-nitroanilino-L- $\alpha$ -aminobutyric acid a deep red, uniform coloration of the cells was obtained (41).

A lowering of the pK of the amino group to 8.2 was obtained in  $\beta$ -chloro-L-alanine (41), but unfortunately this amino acid was dechlorinated in the presence of the tumor cells. It proved also to be an inhibitor of the growth of *Lactobacillus arabinosus* (Table 8); the amino oxidase of *Neurospora crassa* was without effect upon it (47). Other electron-attracting, non-nitrogenous groups are under investigation for their effect upon amino acid transfer into cells.

TABLE 8

INHIBITORY ACTION OF  $\beta$ -CHLORO-L-ALANINE ON DEVELOPMENT OF  
LACTOBACILLUS ARABINOSUS

Chloroalanine solution was sterilized by filtration. Medium slightly modified from that of Schweigert et al. (48). Incubation 72 hours at 30° C. (From P. B. Wilber, T. R. Riggs, and H. N. Christensen, unpub.).

Concentration mM./l.	Acid Production mE./l.
0	195
0.4	118
1.6	65
4.0	21

Such amino acids as triiodothyronine, kynurenine,  $\beta$ -alanine, and taurine are also subject to accumulation (49). The absence of the carboxyl group of an amino acid considerably decreases accumulation, whereas the absence or acylation of the amino group entirely abolishes concentrative uptake (40). Three peptides tested appeared to enter the tumor cells, but only at levels lower than those in the suspending fluid (50).

IX. RELATION OF AMINO ACID ACCUMULATION TO  
POTASSIUM ACCUMULATION

*Mutual inhibition.* The effect of increasing levels of potassium in the suspending fluid on the concentration of glycine by the carcinoma cells is seen in Fig. 13. Inhibition had previously been noted for the rat diaphragm (6) and for erythrocytes (17). In the latter case the effect is particularly striking because of the resistance of the process in erythrocytes to most inhibitors.

The inverse relationship also holds. The presence of a high level of glycine in the extracellular fluid, occasioning the transfer of much glycine into the cell, causes an exodus of potassium, with sodium replacement (19). This effect has been obtained with a number of other amino acids which are strongly concentrated (41). These results are interpreted as indicating a mutual interference between the processes for the transfer of potassium and the transfer of amino acids into the cell.



The exodus of potassium during the accumulation of diamino-butyric acid cannot be cited confidently as a similar situation, because here the action of the law of electroneutrality conceivably may account entirely for the exit of potassium during the entrance of diaminobutyrate. In the competition for concentration the organic

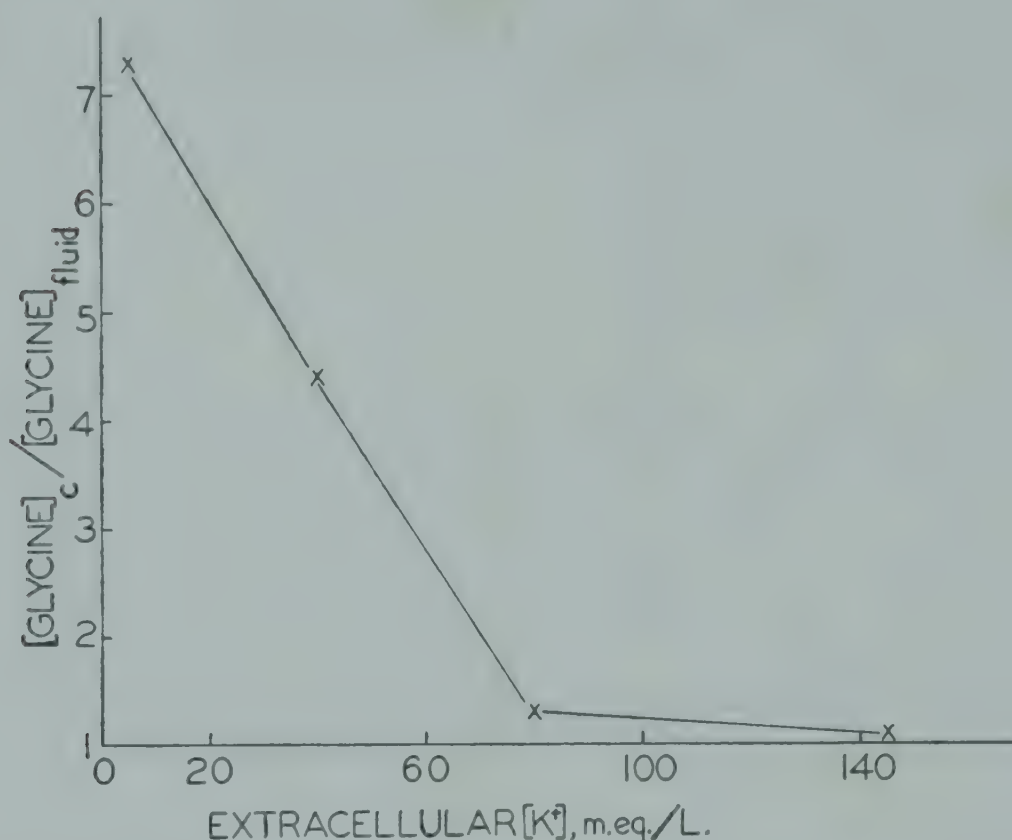


FIG. 13. Inhibitory action of potassium ion on glycine concentration by the ascites tumor cell (18). The accumulation of glycine is also diminished by omitting potassium entirely from the medium.

cation is dominant over potassium (34). Likewise the movement of potassium into the cell along with glutamate appears to be explained adequately by the law of electroneutrality.

*Agents stimulating amino acid uptake cause potassium loss.*

Pyridoxal at levels of 5 to 16 millimolar causes the tumor cells, and also erythrocytes, to lose potassium and chloride. Part of the potassium is replaced by sodium (Fig. 14). If no amino acid is added, the cells shrink to the expected extent. The lower curve of Fig. 9 shows this shrinkage. If sufficient glycine is present, the shrinking is prevented and swelling occurs instead (Fig. 10), because the accumulated glycine more than replaces the osmotic pressure deficit caused by KCl exit. The range of pyridoxal levels which is

stimulating to glycine transfer is extended upward, apparently due to the avoidance of shrinkage.

Most factors which cause potassium to be lost from cells are also injurious to the accumulation of amino acids. Anoxia, lowered temperature, and respiratory inhibitors are examples. Pyridoxal is in fact inhibitory to the respiratory metabolism of the ascites tumor cell (32); the increased glycine accumulation when the oxygen consumption is decreased is a paradox.

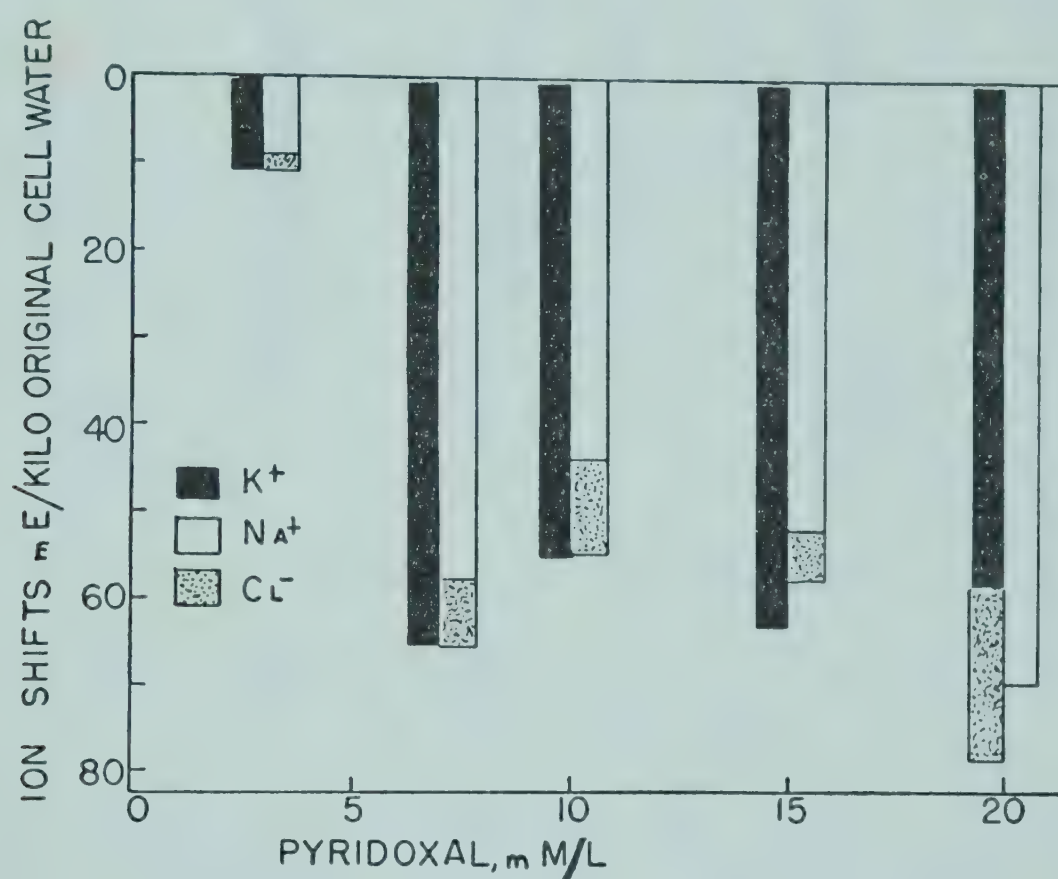


FIG. 14. Shifts of ions of ascites carcinoma cells under the influence of various concentrations of pyridoxal. At the four lower concentrations, the left hand (solid) bar shows the potassium exodus, compared, in the right hand bar, with the entrance of sodium (clear bar) plus the exodus of chloride. Therefore equality of the lengths of the two parallel bars represents the maintenance of electroneutrality, considering these 3 ions. At the 20 mM. pyridoxal level, the net movement of cations is reversed from *outward* to *inward*, and accordingly the chloride movement (now shown in the left bar) is *inward*. (From Christensen, Riggs, and Coyne, *J. Biol. Chem.*, 209, 413, 1954).

The same parallelism in effects upon electrolyte and amino acid distribution is suggested by preliminary experiments with 4-nitrosalicylaldehyde (see Fig. 11). Curiously, members of another group of substances quite unrelated to pyridoxal and which also stimulate glycine transfer, likewise cause potassium and chloride exodus



(Fig. 15). These substances (indoleacetate, phenylacetate, etc.) are required in larger concentrations, and there is no indication that they function in the normal transfer of amino acids. This coincidence of reciprocal effects upon amino acid and potassium transfer might lead one to suspect a simple osmotic reaction, namely, that any substance causing potassium loss would facilitate the uptake of an amino

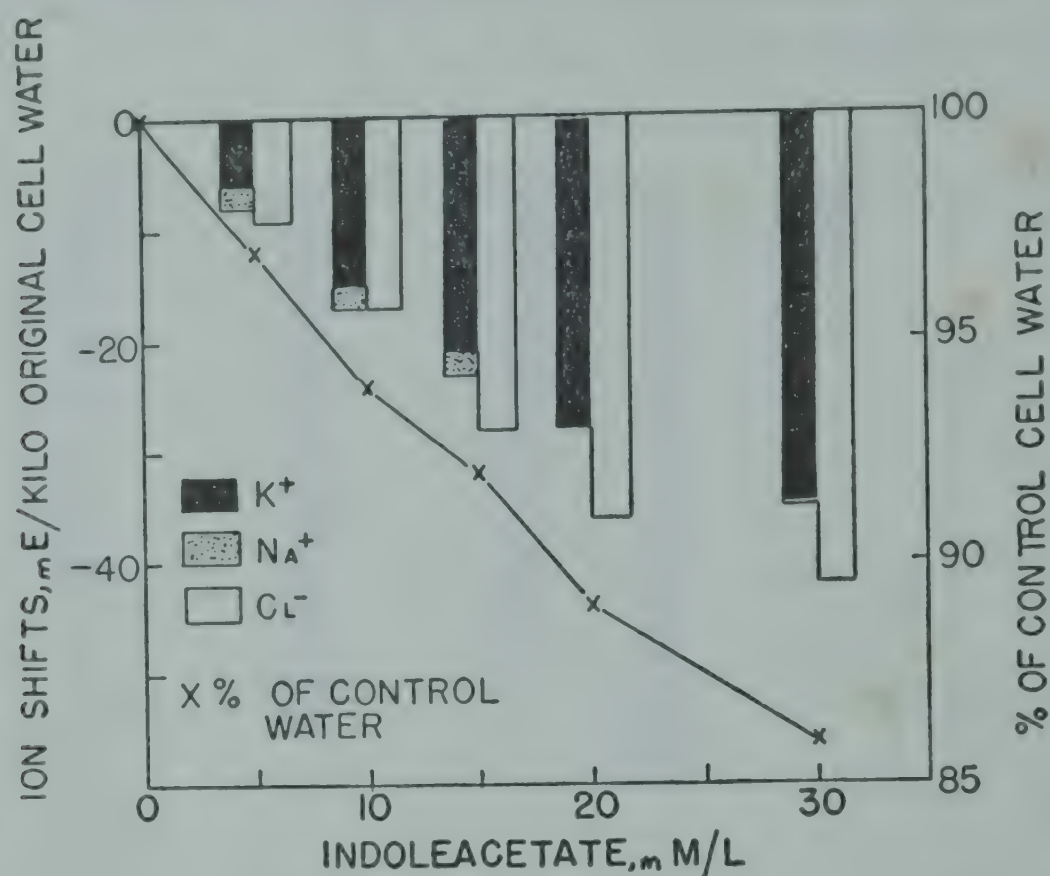


FIG. 15. Loss of ions from ascites carcinoma cells produced by indoleacetate. Cations are shown in the right hand bar, anions in the left hand bar. (From Christensen, Riggs, and Coyne, *J. Biol. Chem.*, 209, 413, 1954).

acid by decreasing the necessity for concomitant water transfer. This is not the case, however, because desoxypyridoxine causes much the same electrolyte shifts as pyridoxal, but it inhibits the accumulation of glycine throughout the range of concentrations tested (32). Nor is there a necessary one-for-one relationship between potassium exodus and amino acid transfer; the potassium loss occurs even if there is little amino acid present to be concentrated. Furthermore, pyridoxal stimulates glycine uptake at low levels of each where osmotic influences are very small and potassium losses are analytically undetectable.

The association between the two processes instead may arise from

the existence of a common reactant or reaction in the two concentration processes, so that diversion from one to the other may occur. One additional possibility to be investigated is that all of these reagents act upon the efflux rates rather than the influx rates. Conceivably they could convert amino-acid-leaky areas of the cell boundary into potassium-leaky areas. In any case the observations indicate that the boundary between high and low potassium levels is probably also the boundary between high and low amino acid levels.

## X. DISCUSSION AND SPECULATION

On the basis of the foregoing evidence the transfer, at least of glycine, into the tumor cells is probably an active transport; that is, glycine is believed to exist at a higher concentration in the interior of the cell than outside the cell. This does not imply that all of the glycine is free in the cell, nor that the volume of distribution of glycine is the whole water content of the cell. There is sufficient similarity in the behavior of almost all other amino acids studied, including especially tryptophan and  $\alpha,\gamma$ -diaminobutyrate (41), to indicate that the modes of their transfer are not entirely different; that is, their transport is probably also active.

Tentatively, it may be suggested that the amino acid exists transiently in a different form, perhaps as a derivative of pyridoxal or of pyridoxal phosphate, in passing through the boundary separating the low and high amino acid levels. In order for the dissociation of such a complex to yield a higher level of amino acid inside the cell than exists outside the cell, energy must be provided. Two ways may be suggested in which this could occur:

1. A compound of higher energy may be split in the formation of the carrier-amino-acid complex, which may then in turn be of sufficiently high energy to release the amino acid at an elevated concentration.

2. A structural change (for example, phosphorylation) of the amino-acid-carrier complex may be produced within the cell to increase its energy content so that the amino acid is dissociated from it at a higher concentration.



One probably cannot yet decide whether accumulation in the gram-positive bacterial cells studied by Gale and his associates proceeds on an entirely dissimilar basis. There are certainly dissimilar elements. The cells we have studied lose their accumulated amino acid if they are washed with saline or distilled water, a procedure routinely applied to the bacterial cells before analysis. Potassium is also retained in the bacterial cells suspended in distilled water. Either the osmotic barrier of the bacteria can restrain the movement of water, or else the potassium and the amino acids of the cell are osmotically inactive. Although nondiffusible forms of potassium have been proposed, the chemical nature of these has never been demonstrated.

According to results of Britten, cited by Gale (8), a part of the glutamate of *Staph. aureus* remains exchangeable even at low temperatures or in the absence of glucose. This exchangeable part is small enough so that it could represent the glutamate in a part of the cell water in a diffusion equilibrium with the extracellular phase; other possibilities for the nature of this fraction may still exist. The active nature of the transport of amino acids into these bacterial cells does not appear to us to be excluded by present evidence. For bacteria, as for cells of higher animals, it seems likely that an understanding of the processes for accumulating potassium and for accumulating amino acids will be achieved together.

A central question seems to be this: Must an amino acid pass through the pool of free amino acids as an essential stage on the way to protein synthesis? Is the activated form in which amino acids are transferred into cells in the direct line of protein synthesis, with the free amino acids as an optional excursion? or must the amino acids be released again to the free form and then be brought into a new activated form?

In the course of the synthesis of proteins the amino acids certainly must enter combinations of a higher energy content; these could release the free amino acids at higher concentrations than those existing in the solution from which they are drawn. Accordingly the protein-synthetic processes can produce an effective concentration of the amino acids, providing the amino acids are drawn



from one compartment (e. g., the extracellular fluid) and released to another one, not too directly connected; and provided also that some high-energy intermediate dissociates sufficiently.

As a special case of this proposition one might suppose that the cellular accumulation of amino acids arises only from the breakdown of the proteins themselves. This is clearly not correct, because upon the addition of a labeled amino acid the free amino acids of the cells become labeled much faster than the protein amino acids; also the accumulated amino acids are known to serve as precursors

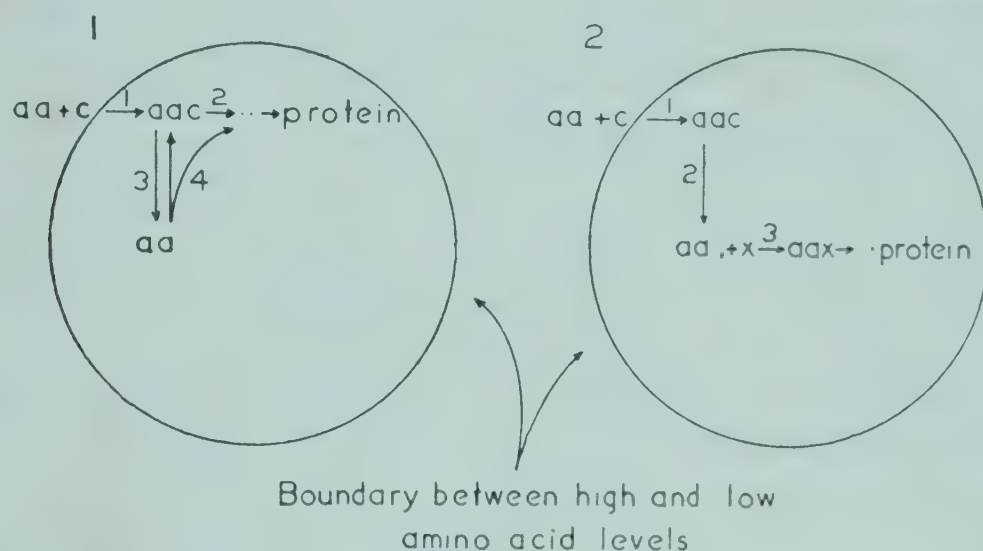


FIG. 16. Schematic representation of possible relationships between the transfer process and protein synthesis. 1. The amino-acid-carrier complex donates the amino acid to other acceptors functioning in protein synthesis. 2. The amino acid must be released and recombined before proceeding into protein synthesis.

of protein synthesis, and accelerate synthetic reactions in the cell (4, 8). Clearly, the cellular free amino acids are not simply "excretory" in nature.

The two alternatives are represented in Fig. 16. In the first alternative a complex is formed between a carrier and the amino acid, which has diffused from the extracellular fluid to an undetermined depth into the cell. This complex passes into the cell interior and is then represented as passing the amino acid directly on to other acceptors (reaction 2) by means of which it enters the peptide chains of proteins. The free amino acids of the cell are formed by a dissociation either of the primary complex or of a subsequent intermediate (reaction 3); these free amino acids must



then be in a compartment which is not in a diffusion equilibrium with the outside fluid. They may then reenter the synthetic process (reaction 4), perhaps by a separate reaction, or perhaps through a spontaneous reversibility of the reaction which released them. In the latter case the difference between the two schemes might actually be of little importance. In any case, the accumulated amino acids are able to accelerate various synthetic reactions which utilize them.

The second scheme represents the amino-acid-carrier complex dissociating to yield the free amino acid (reaction 2), which must then undergo a new activation reaction (step 3) on the way to protein synthesis. The free amino acid of the cell then lies in the direct pathway of protein synthesis.

If the first view is correct, the process we are discussing here is the first step in protein synthesis. A close connection between the vigor of this process and the rate of protein synthesis has already been emphasized. For example, as the reticulocyte matures, it stops incorporating amino acids into proteins and at the same time almost stops concentrating amino acids.

In a number of mammalian tissues, accelerated growth has been observed to be associated with elevated cellular amino acid concentrations. This relationship does not necessarily support either of the two schemes: The protein-synthetic reactions could be accelerated either by the high amino acid levels or by high levels of the precursor (e. g., aa-c) of these free amino acids. In either case the transfer reaction (number 1) seems to be the one that has been accelerated most when protein synthesis is particularly rapid.

It should be mentioned that the situation appears to be somewhat different in the bacteria studied by Gale. Here the size of the actual *accumulations* of glutamate are smaller when protein synthesis is faster. Diversion to protein synthesis appears, at least under some circumstances (See Fig. 7 in Gale and Folkes, 51), to be a large factor in decreasing the amount accumulating in the cell.

One primary need is that the protein-synthetic reactions should not bypass completely the precursors of the various other reactions into which the amino acids enter, for example, transamination,



deamination, and synthesis of heme, purines, epinephrine, creatine, and the like. It is well known that destruction of amino acids for energy takes precedence over the protein-synthetic reactions. This implies that the precursors of the degradative reactions are not depleted or bypassed by the synthetic reactions. It is also well known that in the higher animals the cellular free amino acids are not extensively depleted by starvation. Either scheme can be visualized as protecting the various reactions utilizing amino acids. For scheme 2 this is obvious. In scheme 1 the complex aa-c may be a pyridoxal derivative which can itself serve as a precursor of numerous such reactions; otherwise, reactions 3 and 4 may be related in such a way as to assure the continued presence of a supply of free amino acids in the cell.

Evidence does not seem to be available at the present time for a preference for either of these schemes. Along with the elucidation of the actual transfer process, an understanding of the position of the free cellular amino acids in anabolic reaction sequences seems to be an important objective for research.

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# BIOSYNTHESIS OF $\gamma$ -GLUTAMYL PEPTIDES BY TRANSFER REACTIONS

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SEVERAL MEMBERS of the genus *Bacillus* produce polypeptides composed entirely of glutamic acid which may appear as capsular material, as with *B. anthracis* (5) or as free polypeptide secreted into the medium, as with *B. subtilis* (1). The polypeptide of *B. anthracis* contains only D-glutamic acid (5, 17), while the polypeptide of *B. subtilis* contains from 20 to 75 per cent of its glutamic acid as the D-isomer (17). In the peptides of both species the glutamic acid residues are connected largely or entirely by  $\gamma$ -linkages (1-3, 5, 12).

The production of polypeptide by *B. subtilis* has been investigated in this laboratory (17), and during the course of these studies an enzyme was discovered which catalyzes the hydrolysis of the polypeptide produced by both *B. subtilis* and *B. anthracis*. Further studies have shown that similar enzyme preparations can catalyze a transamidation reaction between glutamine and amino acids or peptides, or with glutamine alone, which results in the formation of  $\gamma$ -glutamyl peptides (19); and a transpeptidation reaction with  $\gamma$ -D-glutamyl-D-glutamic acid<sup>1</sup> which results in the formation of  $\gamma$ -glutamyl peptides of greater chain length (20).

Waelsch has recently reviewed the transfer reactions involving  $\gamma$ -glutamyl compounds (18). These include the transfer of the  $\gamma$ -glutamyl radical from glutamine to various amines by the enzyme glutamotransferase (4, 16), and the transfer of the  $\gamma$ -glutamyl

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<sup>1</sup> The following abbreviations are used: Glu and Asp for glutamic and aspartic acids when these appear in peptide linkage. Thus  $\gamma$ -D-Glu-D-Glu is used for  $\gamma$ -D-glutamyl-D-glutamic acid, etc. Tris = tris(hydroxymethyl)aminomethane, DNFB = dinitrofluorobenzene, DNP = dinitrophenyl, and DPM per  $\mu$ M. = disintegrations per minute per  $\mu$ M.



radical from glutathione to amino acids or peptides to form  $\gamma$ -glutamyl peptides by the enzyme  $\gamma$ -glutamyl transpeptidase (6, 7, 11). Experimental evidence for the transfer of the  $\gamma$ -glutamyl radical from glutamine to amino acids has not been reported in these two systems.

### EXPERIMENTAL

The enzyme was obtained from filtrates of cultures of *B. subtilis* ATCC 9945. The cells were grown in shaken cultures on either a yeast extract-glucose-salts medium (19) or a modified Sauton's medium (20). The enzyme was concentrated by ammonium sulfate precipitation, and in some cases was fractionated by isoelectric precipitation.

The amino acids and biosynthetic peptides were identified by chemical analyses and by qualitative paper chromatography in phenol-water (3:1), propanol-water (4:1), lutidine-water (3:2), and butanol-acetic acid-water (2:1:1). The biosynthetic peptides were isolated by ion exchange chromatography on columns of Dowex 50 resin in the hydrogen cycle, and 0.1 N HCl was used to develop the chromatograms (15). The quantities of di- and tripeptides formed were determined by quantitative paper chromatography (8, 19, 20). Glutamic and aspartic acids were also determined by this procedure. Free amino groups were determined with DNFB (14) according to the procedure of Lowry (13, 19), and DNP derivatives of the peptides were prepared in a similar manner.

### TRANSAMIDATION WITH GLUTAMINE

Incubation of L-glutamine and D-glutamic acid with enzyme resulted in the formation of a new compound which migrated as a dipeptide of glutamic acid on paper chromatograms of the reaction mixture developed in phenol-water. This compound was isolated from large-scale reaction mixtures by ion exchange chromatography, and the peak effluent volume for the new compound was the same as that for authentic  $\alpha$ -glutamylglutamate ( $\alpha$ -Glu-Glu) (about 700 ml.). The compound so isolated migrated on paper chromatograms



as  $\gamma$ -Glu-Glu in phenol-water, lutidine-water, and butanol-acetic acid-water. The butanol-acetic acid-water solvent separates  $\alpha$ - and  $\gamma$ -Glu-Glu and thus permits identification of the structure of the compound. On hydrolysis only glutamic acid was released, as evidenced by paper chromatography in the four solvents used. The glutamic acid released on hydrolysis contained 72 per cent of the L-isomer. That more than 50 per cent of the glutamic acid of this compound was the L-isomer may be explained by the fact that peptide can be formed from L-glutamine alone, and thus some of the dipeptide was probably  $\gamma$ -L-Glu-L-Glu. The compound contained one free amino group in the intact molecule for every 2.3 glutamic acid residues released on hydrolysis. Satisfactory total nitrogen values have been found on preparations of biosynthetic dipeptide, although some of the early preparations contained excess N. These data are consistent with the identification of the biosynthetic compound as  $\gamma$ -Glu-Glu.

Evidence that the reaction may be considered a transamidation reaction in which the  $\gamma$ -glutamyl radical from L-glutamine is transferred to D-glutamic acid was obtained in two experiments. In the first, the dipeptide formed in the above experiment was degraded with DNFB, and it was found that removal of the free amino end-group removed a quantity of L-glutamic acid equal to one-half the total glutamic acid. This shows that L-glutamine contributed the free amino end-group. In the second experiment, L-glutamine was incubated with enzyme and glutamic acid consisting of the D-isomer mixed with a small quantity of DL-glutamic acid-1-C<sup>14</sup> of high specific activity. The peptide formed was isolated by ion exchange chromatography, and the radioactivity incorporated into the compound was determined. The specific activity of the total glutamic acid of the dipeptide was 18,400 DPM per  $\mu M$ . The free  $\gamma$ -carboxyl end-group obtained by degradation of the molecule with DNFB had a specific activity of 37,400 DPM per  $\mu M$ . The crude DNP-glutamic acid representing the free amino end-group contained approximately 400 DPM per  $\mu M$ ., or about 1 per cent of the radioactivity of the free  $\gamma$ -carboxyl end-group. This experiment shows that free glutamic



acid can contribute the free  $\gamma$ -carboxyl end-group but does not appear in significant quantities in the free amino end-group of the  $\gamma$ -Glu-Glu formed.

In two additional large-scale reaction mixtures for isolation and identification of the peptides formed, L-glutamine and enzyme were incubated with L-glutamic acid and with  $\alpha$ -D-Glu-D-Glu. The compound formed in the vessel containing L-glutamine and L-glutamic acid was identified as  $\gamma$ -L-Glu-L-Glu by the procedures mentioned above. Since, as will be shown below, L-glutamic acid does not stimulate dipeptide formation from L-glutamine, the dipeptide formed here must have arisen entirely from L-glutamine.

The compound formed in the vessel containing L-glutamine and  $\alpha$ -D-Glu-D-Glu was eluted from the resin column with a peak effluent volume of 290 ml., which is less than one-half of that found for  $\gamma$ -Glu-Glu. The compound migrated more slowly than  $\gamma$ -Glu-Glu on paper chromatograms developed in phenol-water and lutidine-water, and mixed samples of the new compound with  $\gamma$ -Glu-Glu gave two separate spots. Only glutamic acid was released from the compound on hydrolysis, as evidenced by paper chromatography in the four solvents used. The compound contained 34.5 per cent of its glutamic acid as the L-isomer, and contained one free amino group in the intact molecule for every 3.0 glutamic acid residues released on hydrolysis. These data are all consistent with the identification of this compound as a tripeptide of glutamic acid.

Table 1 shows data from an experiment in which D- and L-glutamine, with or without added D- or L-glutamic acid, were compared as substrates for dipeptide synthesis in this system. Under the conditions of this experiment D-glutamine alone was a somewhat better precursor of dipeptide than was L-glutamine alone. D-Glutamic acid stimulated dipeptide synthesis from L-glutamine more than 2-fold but had no effect on the reaction with D-glutamine. L-Glutamic acid had no effect on the reaction with L-glutamine but inhibited the reaction with D-glutamine about 6-fold. Thus the most active substrates were L-glutamine and D-glutamic acid. In this experiment it was necessary to incubate the vessels for two hours in order to



obtain sufficient dipeptide from the isomers of glutamine alone. In a comparable experiment incubated for only thirty minutes D-glutamic acid stimulated dipeptide synthesis from L-glutamine 4-fold.

TABLE 1

The utilization of optical isomers of glutamine and glutamic acid for dipeptide synthesis. Each vessel contained D- or L-glutamine 40  $\mu M$ , D- or L-glutamic acid 30  $\mu M$ , Tris buffer 20  $\mu M$ , and 0.12 mg. of protein in a total volume of 0.5 ml. The final pH was 8.8, and the vessels were incubated at 36.5° C. for 2 hours.

UTILIZATION OF ISOMERS OF GLUTAMINE AND GLUTAMIC ACID FOR DIPEPTIDE SYNTHESIS		
Optical Isomers of Substrate		$\mu M$ of Dipeptide Formed per ml. of Reaction Mixture
Glutamine	Glutamic Acid	
L-	—	2.6
L-	D-	6.1
L-	L-	2.1
D-	—	3.3
D-	D-	3.4
D-	L-	0.5

The reaction between L-glutamine and D-glutamic acid was proportional to time and to enzyme concentration under the appropriate conditions. Optimal activity was found at about pH 8.8. This enzyme preparation also catalyzed the hydrolysis of L-glutamine. The effect of pH on this reaction was similar to that on the transamidation reaction, and optimal activity was found at about pH 8.8. If the same enzyme catalyzes these two reactions, this effect of pH is different from that on proteases, where the hydrolytic and transfer reactions occur optimally at different pH values (9, 10).

In the reaction between L-glutamine and D-glutamic acid excellent activity was found in the absence of added cofactors, and the addition



of adenosine triphosphate,  $Mn^{++}$ , and phosphate in low concentrations had no effect on the reaction, while 0.04 M. phosphate inhibited the reaction 40 per cent. Iodoacetic acid, N-ethylmaleimide,  $F^-$ ,  $CN^-$ ,  $Cu^{++}$ , and  $Mg^{++}$  all in 0.01 M. concentration were without significant effect on the reaction.

The possibility that the enzyme preparation could catalyze the formation of peptides from L-glutamine and amino acids other than glutamic acid was investigated. Definite evidence of activity with aspartic acid has been obtained. L-Glutamine and DL-aspartic acid were incubated with enzyme, and the reaction mixture was chromatographed on a column of Dowex 50 resin. Prior to the appearance of aspartic acid in the effluent, four ninhydrin positive peaks were eluted from the column with peak effluent volumes of 220, 340, 510, and 750 ml. of solvent. The compounds responsible for these peaks are referred to as compounds A, B, C, and D, respectively. Compounds B and D corresponded on paper chromatography to the tripeptide of glutamic acid and  $\gamma$ -Glu-Glu, respectively. Compounds A and C yielded both glutamic and aspartic acids on hydrolysis and therefore were peptides of these two amino acids. Compound A contained glutamic and aspartic acids in a molar ratio of 2:1, while compound C contained these two amino acids in a molar ratio of 1:1. Both compounds yielded DNP-glutamic acid on degradation by the DNFB procedure, a result indicating that the free amino end-group was glutamic acid. Both glutamic and aspartic acids remained after removal of the free amino end-group from compound A, whereas only aspartic acid remained after removal of the free amino end-group from compound C. These data indicate that compound A is a tripeptide of glutamic and aspartic acids with glutamic acid in the free amino end-group, and that compound C is a dipeptide of glutamic and aspartic acids with a probable structure Glu-Asp.

Since *B. subtilis* produces a glutamic acid polypeptide composed of both D- and L-glutamic acid residues connected largely or entirely by  $\gamma$  linkages, the possibility that this enzyme preparation could catalyze the synthesis of similar polymers from L-glutamine and D-glutamic acid was explored. In this experiment L-glutamine, D-



glutamic acid, and enzyme were incubated for a period of 12 hours, with additions of enzyme and L-glutamine after 4 and 8 hours of incubation. The reaction mixture was chromatographed on paper by the descending technic using phenol-water as solvent. The chromatogram was developed for 7 days. Several spots were found, and these migrated relative to authentic  $\gamma$ -Glu-Glu ( $R_f$  0.10) with  $R_f$  values of 0.10, 0.07, 0.05, and 0.04, and there was an additional spot at the base line which did not move significantly. Larger aliquots of the reaction mixture were streaked on papers and developed in a similar fashion. The compounds were located with guide strips and were then eluted from the paper with water. Only glutamic acid was released from these compounds on hydrolysis. Thus these compounds are peptides of glutamic acid, and their stepwise appearance suggests a stepwise increase in chain length. If this proves to be the case the spots represent a series of peptides beginning with a dipeptide, and proceeding through a tri-, tetra-, and pentapeptide, with possibly even longer peptides remaining at the starting spot.

#### TRANSPEPTIDATION WITH $\gamma$ -D-GLUTAMYL-D-GLUTAMIC ACID

The enzymatic formation of  $\gamma$ -Glu-Glu in the experiments just discussed led to a study of the reactions of  $\gamma$ -D-Glu-D-Glu catalyzed by the enzyme preparations. Incubation of  $\gamma$ -D-Glu-D-Glu with enzyme led to the formation of compounds migrating as glutamic acid and a tripeptide of glutamic acid as detected by paper chromatography of the reaction mixture in phenol-water. A large-scale reaction mixture was incubated and then chromatographed on a column of Dowex 50 resin as before. In addition to the residual dipeptide, compounds corresponding to the tripeptide of glutamic acid discussed above and to free glutamic acid were eluted from the column. The compound believed to be the tripeptide of glutamic acid migrated more slowly than authentic  $\gamma$ -Glu-Glu on paper chromatograms developed in phenol-water, lutidine-water, and fresh butanol-acetic acid-water. On hydrolysis only glutamic acid was released, as evidenced by paper chromatography in the four solvents used. The total N content of the solution of the tripeptide could



be accounted for as glutamic acid N, and one free amino group was present for every 3.5 glutamic acid residues released on hydrolysis. Thus the analytical data are consistent with the identification of this compound as tripeptide of glutamic acid. The compound eluted from the resin column as free glutamic acid migrated as glutamic acid in the four solvents used for paper chromatography.

By using quantitative paper chromatography to measure the tripeptide formed, it was found that with appropriate substrate concentrations the compound believed to be a tripeptide of glutamic acid and free glutamic acid were formed in equimolar quantities at equal rates. With lower substrate concentrations more glutamic acid than tripeptide was formed, a fact which indicates that some hydrolysis of the dipeptide does occur. The release of glutamic acid from  $\gamma$ -D-Glu-D-Glu was proportional to enzyme concentration under the appropriate conditions. Optimal activity for both the release of glutamic acid and the formation of tripeptide occurred at about pH 9.0.

For a more detailed analysis of this reaction with  $\gamma$ -D-Glu-D-Glu, experiments were carried out employing D-glutamic acid-1-C<sup>14</sup>. In the first of these experiments  $\gamma$ -D-Glu-D-Glu was incubated with D-glutamic acid-1-C<sup>14</sup> and enzyme, and after the incubation period the dipeptide remaining was isolated and its radioactivity determined. The glutamic acid released from the dipeptide by acid hydrolysis had a specific activity of 196 DPM per  $\mu$ M. Since, as will be shown below, all of the labeled glutamic acid was present in the free  $\gamma$ -carboxyl end-group of this dipeptide, the specific activity of this moiety was twice that of the total glutamic acid, or 392 DPM per  $\mu$ M. The starting D-glutamic acid-1-C<sup>14</sup> had a specific activity of 10,000 DPM per  $\mu$ M and the exchange was thus about 4 per cent. This exchange reaction suggests the existence of an enzyme-substrate complex as an intermediate in this reaction. That the exchange was only about 4 per cent is possibly due to competition between D-glutamic acid-1-C<sup>14</sup> and  $\gamma$ -D-Glu-D-Glu as replacement agents, since the latter compound is effective in this role as evidenced by the appearance of the tripeptide as a product on incubation of the dipeptide with enzyme.



The labeled dipeptide was degraded with DNFB after it was diluted with unlabeled  $\gamma$ -D-Glu-D-Glu so that the glutamic acid released on hydrolysis contained 72 DPM per  $\mu$ M. The free  $\gamma$ -carboxyl end-group of the dipeptide contained 140 DPM per  $\mu$ M., while the free amino end-group obtained as DNP-glutamic acid was devoid of radioactivity. This shows that the dipeptide was specifically labeled in the free  $\gamma$ -carboxyl end-group by the exchange reaction.

The dipeptide so labeled in the free  $\gamma$ -carboxyl end-group was then incubated with enzyme, and the free glutamic acid and tripeptide which were formed, and the dipeptide which remained, were isolated by ion exchange chromatography. The specific activities of the free glutamic acid and of the glutamic acid released from the peptides by hydrolysis were determined. The glutamic acid of the residual dipeptide contained 71 DPM per  $\mu$ M., the free glutamic acid contained 141 DPM per  $\mu$ M., and the glutamic acid of the tripeptide contained 52 DPM per  $\mu$ M. The specific activity of the dipeptide was unchanged by the incubation, while the specific activity of the free glutamic acid was equal to that of the free  $\gamma$ -carboxyl end-group of the starting dipeptide, and was twice that of the glutamic acid of the dipeptide. The specific activity of the glutamic acid of the tripeptide was 0.73 times that of the glutamic acid of the dipeptide. These data are all consistent with the reaction mechanism given in Fig. 1.

Equation *A* is for the enzymatic exchange reaction between D-glutamic acid-1- $C^{14}$  and  $\gamma$ -D-Glu-D-Glu, and equation *B* is for the reaction of  $\gamma$ -D-Glu-D-Glu with the proposed enzyme-substrate complex. The overall reaction is given by equation *C*. Here 2 moles of dipeptide react to yield one mole of free glutamic acid, which is labeled and one mole of tripeptide containing one labeled glutamic acid residue. From this overall equation the ratio of specific activities of the free glutamic acid to the glutamic acid of the dipeptide would be 2, and the ratio of the specific activities of tripeptide glutamic acid to dipeptide glutamic acid would be 0.67. These are the ratios found experimentally. Thus the reaction appears to be a transpeptidation in which the free amino end-group of one mole of dipeptide is trans-



ferred to a second mole of dipeptide to form a mole of tripeptide containing one labeled glutamic acid residue, and the free  $\gamma$ -carboxyl end-group is released as free glutamic acid.

PROPOSED MECHANISM OF ENZYME ACTION WITH LABELED  
 $\gamma$ -D-GLUTAMYL-D-GLUTAMIC ACID AS SUBSTRATE

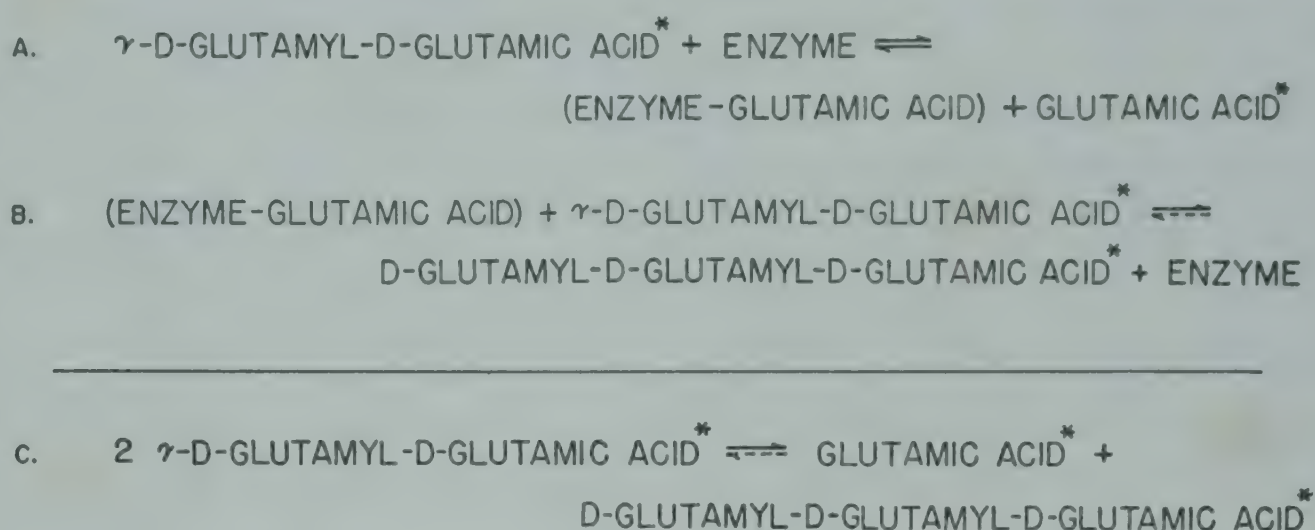


FIG. 1. Proposed mechanism of enzyme action with labeled  $\gamma$ -D-glutamyl D-glutamic acid as substrate.

In experiments to determine whether peptide chains longer than three glutamic acid residues can be formed from  $\gamma$ -D-Glu-D-Glu in the presence of enzyme, high concentrations of both enzyme and substrate were incubated for 4 hours. A descending paper chromatogram of the reaction mixture was developed in phenol-water for 7 days. A series of spots similar to that found in the experiment with L-glutamine and D-glutamic acid recorded above was found for this reaction mixture. Here spots corresponding to the di- and tripeptides of glutamic acid were present, and in addition there were two spots migrating more slowly than the tripeptide and some ninhydrin-positive material remaining very near the starting spot. Thus the paper chromatographic evidence indicates that longer peptides may be formed from  $\gamma$ -D-Glu-D-Glu as well as from L-glutamine and D-glutamic acid.



## SUMMARY

An enzyme preparation obtained from filtrates of cultures of *B. subtilis* catalyzed a transamidation reaction in which the  $\gamma$ -glutamyl radical was transferred from L-glutamine to D-glutamic acid,  $\alpha$ -D-Glu-D-Glu, aspartic acid, and probably to glutamine itself to form glutamyl di- and tripeptides. The dipeptides of glutamic acid were  $\gamma$ -linked. Both L- and D-glutamine were active as substrates in this system, but only the D-isomer of glutamic acid was active as a replacement agent. More dipeptide was formed from L-glutamine and D-glutamic acid than from the other combinations of the optical isomers of these two compounds. No added cofactors were required for the reaction. Optimal activity was found at about pH 8.8. The enzyme preparation also catalyzed the hydrolysis of L-glutamine, and optimal activity for this reaction occurred at about pH 8.8.

The enzyme preparation also catalyzed a transpeptidation reaction with  $\gamma$ -D-Glu-D-Glu which resulted in the formation of free glutamic acid and a compound believed to be a tripeptide of glutamic acid. This reaction could proceed almost entirely as a transfer reaction. No added cofactors were required, and optimal activity for the formation of both glutamic acid and the tripeptide was found at about pH 9.0. Experiments bearing on the mechanism of the reaction were performed using D-glutamic acid-1-C<sup>14</sup>, and these are discussed.

From L-glutamine and D-glutamic acid or from  $\gamma$ -D-Glu-D-Glu the enzyme catalyzed the synthesis of a series of compounds, as evidenced by paper chromatography, which were probably glutamic acid peptides of increasing chain length. The possibility exists that a polypeptide similar to that produced by *B. subtilis* may be synthesized by this enzyme preparation. Further studies to determine whether this can actually occur are under way.

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## DISCUSSION

DR. GALE: I agree with Dr. Christensen that it would be very nice if we could come to a common explanation of the transfer phenomena for all types of cell, but I think that, at the moment, any such supposition would be extremely dangerous and that we should recognize differences from cell to cell. There are many qualitative and quantitative differences between the findings made by Christensen with carcinoma cells and those made by Spiegelman with yeast and our own findings with staphylococci. The ultimate basic mechanism may be the same in all cases, but the superficial picture differs in each case. Of course, there are large structural differences in the cells being studied; staphylococci and yeast cells have a rigid, strong membrane surrounding them and are not susceptible to the volume changes described by Christensen in carcinoma cells. We know that the staphylococcal cell can withstand very high osmotic pressure; work which is being carried out by Dr. P. D. Mitchell at Cambridge indicates that the staphylococcus has an internal osmotic pressure of 15 to 20 atmospheres. I do not know whether this has any relation to some of the differences that have been described for the free amino-acid pool—differences such as are shown by washing in water which removes amino acids from carcinoma cells but does not remove them from either staphylococcal or yeast cells.

The accumulation of amino-acids by staphylococci and yeast requires the provision of energy; this can be demonstrated readily and unequivocally. One of the fundamental problems which we have been thinking about for a long time is: where in the cell is the energy required? Is it required to



transport amino-acids across an impermeable membrane, or is it required for some sort of metabolic binding inside the cell? I do not think the situation is quite as clear now as it seemed a few months ago. At that time we received a report from Washington, and I hope Dr. Roberts will have time to report on this, which showed that an exchange of labelled glutamic acid will occur across the staphylococcal cell wall, an exchange apparently due to a diffusion process. Such diffusion of glutamic acid without energy requirement took place into 30-40% of the cell volume. Recently Mitchell in Cambridge has devised a method for determining the impenetrable volume of micro-organisms for various metabolites; he has, for example, published results showing the impermeability of staphylococci to phosphate. Applying his methods he has recently found that the impenetrable volume of the staphylococcus to free glutamic acid is of the order of 90% of the cell volume; in other words, within experimental error all of the *Staphylococcus* is impenetrable to glutamic acid. Consequently we have returned to our earlier theory that the accumulation of free glutamic acid by *Staph. aureus* involves active transport of the amino-acid across a cell membrane.

One other point, that I would like to bring up to emphasize the differences between our work on staphylococci and that of Dr. Christensen on carcinoma cells, concerns the relation of free amino-acid accumulation to protein synthesis. In staphylococci, accumulation of free amino-acids is minimal during protein synthesis and is maximal when protein synthesis is not taking place or is inhibited. When protein synthesis is going ahead strongly there is little or no accumulation. This is not just a question of the glutamic acid in the cell being diverted into protein synthesis, since the rate at which glutamic acid is withdrawn from the medium is much greater in the absence than in the presence of protein synthesis. During protein synthesis, glutamic acid is taken up into protein promptly and quantitatively and there is no accumulation of the free amino-acid in the pool inside the cell. Disorganize protein synthesis in any way you please and accumulation of free glutamic acid begins while the rate of its uptake from the external medium increases. This is quite different from the findings Dr. Christensen has described in carcinoma cells. I suggest—and this is sheer speculation—that we might consider the possibility that there is a membrane near the surface of the cell, that this membrane is impermeable to certain free amino-acids but possesses certain activities, one of which results in the transport of these amino-acids across the barrier, and another results in the synthesis of protein so that both processes go on near the surface of the cell.

DR. ROBERTS: I would like to leave any discussion of the permeability of *Staphylococcus* to Dr. Britten; but I would like to say a few words about the situation in *E. coli*, which has quite a bit of similarity to some of the carriers that Dr. Christensen mentioned. *E. coli* seems to be completely permeable to



amino acids. They go in freely and also leave the cell freely, and yet during the growth of *E. coli* with glucose as an energy source the amino acids do not leak out into the medium. So we already have an indication that there is some sort of binding or carrier in the amino acid metabolism. This becomes much more clearly demonstrated when you think of some of the particular sequences of amino acid synthesis. In the case of threonine, for example, the pathway is from aspartic to homoserine, then to threonine, and then on to isoleucine. This would be a rough sketch of the flow of carbons. We can label threonine by growing the cells in the presence of 1- $C^{14}$ -glucose,  $C^{14}$ -acetate,  $C^{14}O_2$ , or  $C^{14}$ -aspartic acid. All of these tracers will result in the labelling of threonine, which ends up in the protein containing  $C^{14}$ . All of the above tracers also label the isoleucine. I should add at this point that these tracers do not lead to  $C^{14}$ -glycine, so we have evidence here that internally synthesized threonine is not being converted to glycine. On the other hand, if we add  $C^{14}$ -threonine from the outside it does reach the site of protein synthesis and appears as  $C^{14}$ -threonine in the protein, as if very little internally synthesized threonine is being made. The external threonine also goes on to isoleucine, so it clearly reaches the site where threonine is reacting, but at the same time a very large quantity goes to glycine. Here we have therefore a case where the cell clearly distinguishes between internally made threonine and externally supplied threonine. You can try to construct permeability barriers within the cell to shield some of these reactions, but you find that you have to include in that permeability barrier all the sites at which threonine is being used; so it becomes very difficult to think of any permeability barriers. The only alternative then is to consider that within the cell the intermediates—such as aspartic acid and homoserine—are attached to some R group as they proceed through the various reactions and are finally incorporated into proteins. Otherwise the cell could not prevent the internally synthesized threonine from being converted to glycine.

DR. BRITTEN: We have studied the exchange of external  $C^{14}$ -glutamate and internally stored  $C^{12}$ -glutamate in staphylococcus cells. After their capacity to store glutamate is saturated at an external concentration of 0.02 molar glutamate, we observe an exchange rate at 30° C. (in the absence of glucose) of 20 micromols per hours per gram wet cells. This exchange rate is temperature dependent and falls to zero at about 5° C. Since the concentrations of internal and external glutamate remain constant, no net energy is required for this process. In addition we observe an immediate and temperature-independent exchange of an amount of glutamate corresponding to 35 per cent of the volume of the cell pellet at the external concentration of glutamate. The fraction of the volume of the pellet available for immediate exchange is independent of the concentration of the external glutamate. It is almost identical to the immediately accessible volume measured by Dr.



Mitchell for phosphate ions by a similar technique. Dr. Mitchell interprets this volume as principally intercellular fluid. It appears to me that, at most, a small fraction of the internal cell volume is available for immediate free exchange of phosphate or glutamate ions.

DR. CHRISTENSEN: I am particularly anxious to learn about these determinations by Dr. Britten, because they constitute one of the main reasons for drawing a large difference between the bacterial and the mammalian cell. The fact that the amino acid exchange goes on at low temperatures in the absence of an exogenous energy source is not enough to rule out active transport. Nor is the finding that the quantity of glutamate which remains exchangeable under these conditions is equal to or smaller than the amount which would be present in the cell water were it in diffusion equilibrium with the external environment. Those things together do not establish that only diffusion is going on. Therefore, I would like to ask Dr. Britten whether there are further criteria that the process of exchange which goes on at low temperature in the absence of glucose actually is a diffusion equilibrium involving an important part of the cell water.

DR. BRITTEN: I don't believe we have any evidence in *Staphylococcus* cells for diffusion equilibrium occurring with any large part of the cell volume. The situation is quite different in *E. coli* where 75 per cent of the cell volume is freely accessible to small molecules.

DR. CHRISTENSEN: The sort of process described by Dr. Roberts for threonine may occur generally; that is an amino acid bound to a catalytic system may well have a different fate than exogenous amino acids. The difference is that the amino acids I have referred to largely are *not* bound to the catalytic systems of the cell. Of course we cannot exclude that these "free" amino acids may include some catalyst-bound portions, but the amount cannot be appreciable because of the low concentration of such catalytic systems. The evidence for the mainly free state of the amino acids in these cells has not seemed to us easily refutable. Whether the exchange for other cells occurs by a process of diffusion is a matter subject to direct investigation.

DR. HALVORSON: I should like to comment on several features of the free amino acid pool in yeast especially as they compare with their parallel pools in *E. coli* and mammalian cells. One observation of considerable interest was the apparent independence of the composition of the pool in yeast from the nature of the external environment and also of the medium on which the cells are grown. The only success that we have had in varying the pool rather remarkably is in the case of a lysine-deficient mutant. This mutant accumulates lysine rather remarkably during the exponential phase of growth. The lysine constant then drops as the growth ceases. The behavior of free lysine is not seen in the other members of the free amino acid pool.



A second point of comparison between pools of various organisms is evidenced by the coupling between pool accumulation and protein synthesis. We have stopped the synthesis of protein by several means, including ultraviolet light and amino acid analogues and find that pool accumulation and interconversion continues. I think one need not have a mandatory accompaniment of protein synthesis in yeast in order to have amino acid accumulation. In this respect yeast cells show many of the properties of *Staphylococcus aureus*. The last comment I should like to make relates to the comment Dr. Christensen made that the free amino acid pool may not represent a direct precursor between exogenous amino acids and cellular proteins in mammalian cells. A few years ago Dr. Spiegelman and I found that we could inhibit protein synthesis and adaptive enzyme formation in yeast by amino acid analogues. That has been extended both to several different enzyme systems and different organisms. These analogues act in a competitive fashion, being completely reversed by their homologous amino acids. The simplest explanation, I believe, for the kinetics and specificity in their behavior would be that the free amino acid pool represents a precursor in protein and enzyme synthesis.

DR. ADELBERG: I would like to comment on Dr. Roberts' explanation of the ability of the cell to distinguish between endogenous and exogenous compounds. There are other examples of this. For example, when tryptophan is being made endogenously there is virtually no formation of the kynureninase system, but if you put the same organisms in the presence of an exogenous supply of tryptophan there is an induction of the kynureninase system and therefore a different metabolism of tryptophan occurs. I am wondering whether this simply couldn't be a difference in the concentration of the compound. When you put threonine in the medium there is enough to induce the formation of the enzyme, which splits threonine to glycine. There may not be any difference in the state of the carrier system.

DR. ROBERTS: No. This happens in the first ten minutes, and it happens with tracer amounts of threonine and carrier-free threonine. It is less than a microgram.

DR. WORK: In my laboratory, Dr. Hoare has examined some of the free amino acids of *Sarcina lutea* grown on different protein hydrolysates. With peptone broth as the growth medium, a high concentration of hydroxyproline was found in the soluble amino acid fraction. When we changed to casein hydrolysate, which contains no hydroxyproline, there was no hydroxyproline in the cells. Evidently in this organism, the composition of the amino acid pool varies with the growth medium—at any rate with regard to hydroxyproline.

DR. CHRISTENSEN: In this connection, I have not elaborated on the very wide variety of synthetic structures which have no part in metabolism, which are accumulated as long as they have the amino acid structure. Some of these



are a good deal more strongly accumulated than the naturally occurring amino acids. An interesting case is the behavior of  $\alpha$ -aminoisobutyric acid in the whole animal. Because of its accumulation by the tissues this structure is excreted quite slowly from the animal organism.

DR. L. MILLER: With respect to Dr. Christensen's discussion, it would be interesting to know what the actual concentration of pyridoxine in the cell is compared to the concentration he found necessary to effect this accumulation or transport process.

DR. CHRISTENSEN: I think this is an important consideration. If we are to consider a possible coenzyme-like function of pyridoxal in the transfer process, we need to explain the rather substantial amounts required to produce a maximal stimulation. This requirement might be explained if pyridoxal is only a precursor of the carrier, or it might be explained by the difference in the function of a "carrier" from most other coenzymes or cofactors. Presumably the carrier-substrate complex must dissociate from a macromolecular catalytic system upon which it is formed, to enter into the cell, there to be dissociated to yield the free amino acid by a second enzymic reaction. In contrast, in other enzymic reactions the coenzyme probably may remain in association with the apoenzyme throughout its function; therefore relatively small amounts of coenzyme may be required to saturate the system. But in a transfer system the carrier needs to be kept at a concentration higher outside than inside the cell. In this type of system much larger amounts of cofactor might be required for saturation.

DR. KAPLAN: I was wondering if the enzyme splits hydrolytically D-glutamine.

DR. WILLIAMS: Yes. It will hydrolyze both D- and L-glutamine. In the presence of L-glutamine and D-glutamic acid about  $\frac{2}{3}$  of the glutamine reacts in the transfer reaction to give dipeptide and about  $\frac{1}{3}$  is hydrolyzed, but if you put in glutamine alone it is largely hydrolyzed and only a small quantity yields dipeptide.

DR. STRECKER: When you use L-glutamine and D-glutamic acid, do you obtain the L,D- or the D,D-peptide?

DR. WILLIAMS: With L-glutamine and D-glutamic you get a mixed peptide containing about 72 per cent L, indicating that about half of it would be L-glutamyl-D-glutamate and the other half would be L-glutamyl-L-glutamate. These are rather long incubations which we use for isolation of the compounds and apparently during this time enough L-glutamine can react with itself to form the L-glutamyl-L-glutamate found.

DR. LIPMANN: Can you form glutamine by reaction of the dipeptide with ammonia?

DR. WILLIAMS: We haven't tried that yet, but we expect to look into that in the near future. We hope it will work.



# FREE AMINO ACIDS AND THE ENZYME-FORMING MECHANISM

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## I. INTRODUCTION

IT HAS BEEN established in a variety of cases (see 43, 59, 62 for recent reviews) that "enzymatic adaptation" in microorganisms involves the synthesis of new enzyme molecules rather than the activation of preexistent non-functional precursors. Evidence which will be briefly reviewed in the present paper demonstrates further that this synthesis is a process *de novo* from the constituent amino acids. This evidence equates induced synthesis of enzymes with the problem of the formation of at least a particular class of proteins. The possibility of following the increase of these proteins by means of their enzymatic activity increases enormously the range of performable experiments in terms of both sensitivity and variety.

Some may argue that the inducible enzymes represent a class so unique in their properties as to preclude their serving as suitable models of protein synthesis. One cannot offer definitive data categorically denying the validity of this argument. We feel, however, that it represents but a remote possibility. The fact that one and the

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same enzyme can be "constitutive" in one strain and inducible in another argues against a uniquely and radically different mechanism of synthesis for the inducible enzymes. In any event, it seems difficult to believe that the information obtained from the study of the induced formation of enzymes will not be relevant to the problem of protein synthesis in general.

Any attempt at unraveling the mechanism of enzyme formation, using inducible systems, resolves itself quite naturally into attempts to provide adequate answers to the following questions:

(a) What is the role of the inducer, the presence of which specifically stimulates the formation of the corresponding enzyme?

(b) What is the nature of the precursor material which is transformed into active enzyme molecules?

(c) What is the nature of the enzyme-forming mechanism which converts the precursor material into active enzyme?

In view of the purpose of the present conference we shall confine our attention primarily to the second question. The results obtained on the precursor problem possess definite implication for the nature of the enzyme-forming mechanism. These will be discussed along with a description of some experiments which they suggest.

The problem of the precursor is most dramatically exhibited by induced synthesis of enzyme carried out in the absence of an external nitrogen source. In such inductions the nitrogen employed by the cell in fabricating the new enzyme molecule must derive from some preexisting nitrogenous compounds in the cell. One is immediately faced with the obvious necessity of identifying the components thus employed. Ability to form enzyme in the absence of an external supply of nitrogen is far more widespread among the yeasts than among the bacteria. The work of Taylor (64) suggests a reasonable explanation for this apparent independence of the yeast enzyme-synthesizing mechanism. Employing the amino acid decarboxylase procedure (21, 65), Taylor surveyed a variety of yeasts and bacteria for the presence of free amino acids in their internal environment. Analyses were made for arginine, glutamic acid, histidine, lysine, and tyrosine. Of the three yeast types examined, all possessed



detectable quantities of these five amino acids. Amongst the bacteria the gram positives possessed primarily glutamic acid and lysine. None of the gram negatives included in the survey contained detectable free amino acids by the procedures employed.

It would appear that the ability of yeasts to get along without an external source of nitrogen in enzyme synthesis is due to the fact that they have an internal supply. These findings possess evident implications for the proper analysis of the enzyme precursor problem in yeasts. A prerequisite for the use of this material is an adequate understanding of the free amino acid pool and knowledge of methods which would permit the exercise of experimental control over its level and composition. Accordingly, a fairly extensive examination was instituted of the properties and behavior of the pools in yeasts. It seems appropriate here to preface our major concern, which is the synthesis of enzymes, with a discussion of the information accumulated. In so doing we will avoid detailing data which have already been published, and focus our attention on information which has not yet appeared in print.

## II. THE PHYSIOLOGY AND PROPERTIES OF THE FREE AMINO ACID POOL

### 1. *Methodology*

In designing methods for obtaining pool samples we were primarily guided by the researches of Gale (22). The procedure which gave us the most consistent results, and was at the same time the simplest, was the boiling method. In this, a yeast suspension (10 per cent by volume of cells) is placed in a boiling water bath for ten minutes and then cooled. The material is then centrifuged and the supernate retained for analysis. The quantitative values thus obtained agree excellently with those from other methods which are in principle much gentler. Thus, another procedure examined uses "fast dried" cells. Here the cells are washed by centrifugation and placed in a vacuum dessicator over drierite, and continuous pumping is maintained for four hours. The dried cells are then extracted with water for 30 minutes in the cold. The debris is

removed by centrifugation and the supernate retained for analysis. Table 1 compares the values obtained for free pool levels by these two procedures in terms of the glutamic acid content. Identical results are obtained with the two procedures. It should be noted that the data exhibited in Table 1 were checked and confirmed for other pool components by inspection of amino acid chromatograms (42). Other methods, including alumina grinding and rupture with toluene, have given consistently lower values.

TABLE 1  
COMPARISON OF POOL SAMPLING PROCEDURES  
GLUTAMIC ACID ANALYZED BY THE DECARBOXYLASE METHOD

Method of Preparation of Sample	$\mu$ M. Glutamic Acid/100 mg. dry cells	
	Samples at zero time	120 min. N-starved
15 min. Boiling	8.9 $\pm$ 0.14 *	2.2 $\pm$ 0.11 *
Fast Drying	8.80 $\pm$ 0.16 *	2.0 $\pm$ 0.14 *

\*  $2\sigma_m$  (standard deviation of the mean).

TABLE 2

COMPARISON OF BOILING TIME IN PREPARING FREE AMINO ACID POOLS

A suspension of harvested and washed cells was suspended in water to a density of 28.4 mg. dry cell/ml., placed in boiling water for various periods of time, removed, cooled, and centrifuged. Supernatants were assayed for free amino acids by decarboxylase methods.

Time of Boiling	$\mu$ M./100 mg. dry cells	
	Glutamic Acid	Lysine
2	12.8	12.1
5	12.6	11.3
10	13.2	11.7
15	12.8	11.9
20	12.7	11.9

It has been found that the time of boiling is not critical. This is well illustrated by Table 2, which compares the amounts of glutamic acid and lysine found in free pool preparations obtained after various periods of boiling. It will be noted that within two minutes



the maximal levels of both amino acids are observed in the supernatant, and more extensive boiling does not result either in their destruction or in the breakdown of any existing components which can yield either one of these two amino acids.

In view of its simplicity and consistency, the boiling procedure has been employed throughout the investigations to be described in the analysis of the free amino acid pools.

## 2. Pool Composition

### A. Survey of strains.

The observations of Roine (51) and Taylor (64) on the existence of free amino acids pools in yeast were readily confirmed with strain K of *Saccharomyces cerevisiae*. Examination (34) of the free pool content of exponential phase cells, grown in complete medium, revealed that of 16 amino acids analyzed for by microbiological methods, all could be found in detectable amounts. The major components were glutamic acid, aspartic acid, and serine.

It was of some interest to see how extensive the possession of considerable free pools was amongst the yeasts. A survey was made

TABLE 3

#### SURVEY OF FREE AMINO ACID POOLS IN A VARIETY OF YEAST STRAINS

Cells were grown in complete medium and harvested at the end of the log phase. Pools were analyzed for glutamic acid (GA) and total nitrogen content.

Strain	$\mu$ M. of GA/100 mg.	mg. N/100 mg. dry cells	Total mg. pool N/100 mg. dry cells	% pool N as GA
P. strain A (haploid)	23.5	.225	1.13	19.9
R 427a (haploid)	23.8	.228	1.342	17.0
R 427A (haploid)	21.1	.210	0.743	28.6
L 1428A (haploid)	18.2	.174	1.92	9.05
<i>S. cerevisiae</i> (strain K)	13.8	.132	0.505	26.1
<i>S. carlsbergensis</i> (Y-379)	10.2	.096	0.781	12.3
<i>S. carlsbergensis</i> (Y-1005)	10.3	.097	1.038	9.35
<i>S. fragilis</i> (VN)	11.2	.107	1.13	9.5
<i>S. fragilis</i> (Y-1342)	12.3	.118	1.24	9.5
<i>S. chevalieri</i>	13.2	.126	1.24	10.2
<i>S. ludwigii</i>	14.1	.135	0.921	14.6
<i>S. italicus</i> (Y-1434)	16.4	.157	1.67	9.4

of different yeast species as well as of different representatives of *S. cerevisiae*. The results obtained are summarized in Tables 3 and 4. The first four strains listed in both tables are haploids, and the remainder are diploid varieties. It is apparent that all of the strains examined possess considerable amounts of free amino acids, extending from about 5 to 12 per cent of the nitrogen of the cells.

TABLE 4  
FREE AMINO ACID POOL COMPOSITION OF VARIOUS YEAST STRAINS AS  
DETERMINED BY PAPER CHROMATOGRAPHY

Strain	Amino Acids												
	1. Alanine	2. Serine	3. Glycine	4. Leucine	5. Threonine	6. Glutamic Acid	7. Aspartic Acid	8. Glutamine + Alanine	9. Lysine	10. Threonine	11. Arginine + Valine	12. Tyrosine	13. Hydroxyproline
P. strain A	+++	++	++	+++	+	++	++	++	+	+	++	+	-
R 427a	++	++	+	++	+	+++	++	++	trace	trace	++	+	+
R 427A	+++	++	+	+++	+	+++	++	++	+	+	++	-	-
<i>S. cerevisiae</i> sex A	++	++	++	+++	+	++	++	+++	+	+	++	+	-
<i>S. cerevisiae</i> (strain K)	+++	++	++	+++	+	+++	++	+++	+	+	++	+	-
<i>S. carlsbergensis</i> (Y-379)	+++	++	++	++	++	+++	++	+++	+	+	-	-	-
<i>S. carlsbergensis</i> (Y-1005)	+++	+	++	++	+	+++	+	+++	+	+	+	-	-
<i>S. fragilis</i> (VN)	+++	++	++	+++	+	+++	+	++	+	+	-	-	-
<i>S. chevalieri</i>	+++	++	++	+++	++	+++	++	++	+	+	+	+	-
<i>S. ludwigii</i>	+++	++	trace	++	+	+++	++	+++	+	trace	+	-	-
<i>S. italicus</i> (Y-1434)	++	+	+	++	+	++	++	++	trace	+	-	+	-

Strikingly unique patterns of pool composition were not detected in the course of this examination. Quantitatively, the only consistent picture to emerge is the apparent high pool content of the haploid stocks.

B. Effect of growth medium on pool composition.

Nagai (46) has found that growth of yeast in media containing NaCl at hypertonic levels leads to the disappearance of many free



amino acid pool components. Only glutamic acid, alanine, histidine, and small amounts of aspartic acid are found in such cells.

Table 5 shows that pools of somewhat modified composition can be obtained by growing the organisms in media with differing carbon sources. It will be noted that with glucose as the major carbon source,

TABLE 5

COMPOSITION OF THE FREE AMINO ACID POOL OF *Saccharomyces cerevisiae* STRAIN K GROWN ON DIFFERENT CARBON SOURCES AND DETERMINED BY PAPER CHROMATOGRAPHY

Cells were harvested near the end of the logarithmic phase.

Amino Acid	Glucose Broth	Glucose Synth. Medium	Pyruvate Synth. Medium	Lactate Synth. Medium	Glycerol Synth. Medium
Aspartic Acid	++	+	+++	++	+
Glutamic Acid	+++	++	+++	+++	+++
Serine	++	++	trace	trace	trace
Glycine	++	+	trace	trace	trace
Glutamine + Alanine	+++	++	+++	++	+++
Tyrosine	++	+	++	trace	trace
Threonine	+	+	+	trace	trace
Lysine	+	+	+	trace	trace
Valine	++	+	++	+	+
Leucine	++	+	++	+	+

relatively little difference is observed between cells grown in complete or synthetic medium. However, several of the amino acids almost disappear from the pools of cells grown on either pyruvate, lactate, or glycerol. The almost complete absence of serine and glycine in these cases may find explanation in the primarily aerobic mechanism involved in the utilization of these compounds. A consequence could well be a greater demand for porphyrin synthesis.

### 3. Depletion and Replenishment of Free Amino Acid Pools

#### A. Starvations and replenishments with glucose as the carbon source.

One of the most successful methods found for varying both pool level and pool composition was the employment of cycles of nitrogen depletion and replenishment (33, 51). Pool levels were readily

lowered by exposing cells to a nitrogen-free medium containing glucose. Restoration of the amino acid pools could be achieved by subsequent exposure of the starved cells to a medium containing a nitrogen source and glucose. The quantitative effects on eleven of the amino acids in the pool are depicted in Table 6. These data

TABLE 6

A COMPARISON OF FREE AMINO ACID POOL COMPONENTS OF UNSTARVED, STARVED, AND REPLENISHED CELLS

Starvation was carried out by incubation in a nitrogen-free 2%-glucose synthetic medium for 12 hours at 30° C. Replenishment was accomplished by 15 min. incubation in the same medium containing either 1% NH<sub>4</sub>Cl or 0.5% enzymatic digest of casein. The free amino acid pools were analyzed microbiologically.

Amino Acid	$\mu$ M./100 mg. cells			
	Unstarved	Starved	Replenished (casein digest)	Replenished (NH <sub>4</sub> Cl)
Valine	1.57	0.68	2.36	1.40
Glutamic acid	10.0	3.10	15.6	10.9
Aspartic acid	3.6	0.62	3.30	2.70
Histidine	0.97	0.15	0.43	0.21
Lysine	4.3	1.78	7.9	1.76
Isoleucine	0.64	0.35	1.26	0.51
Proline	0.92	0.15	0.83	0.26
Serine	3.67	1.9	6.9	3.0
Methionine	0.60	0.13	0.54	0.14
Threonine	2.20	0.64	1.20	0.82
Arginine	1.20	0.36	2.07	0.58

reveal that the starvation procedure leads to the decrease of all pool components. They also demonstrate the possibility of varying the extent of the replenishment by modifying the nitrogen source employed and the extent of the replenishment procedure. Thus, with the casein digest, virtually all components are restored to the original unstarved levels, or to levels exceeding these. On the other hand, when NH<sub>4</sub>Cl is employed several components (e. g., methionine, threonine, arginine, proline, lysine, histidine) are not restored to normal levels, and in some cases are not increased by the replenishment procedures. It should be noted that a more extensive exposure to NH<sub>4</sub>Cl does restore all components to normal levels. In any case,



such devices permit one to examine the effects of varying pool levels on enzyme synthesis both during the exhaustion of the free amino acid pool and during its replenishment.

*B. The use of other energy sources for pool depletion.*

It was desirable for a variety of reasons to examine in further detail other conditions which might permit control over pool levels. It was quickly revealed that supplying an energy source in a nitrogen-free medium was not a sufficient condition for the depletion of the free amino acid pool. This is illustrated by the data in Table 7.

TABLE 7

COMPARISON OF GLUCOSE AND ETHYL ALCOHOL AS ENERGY SOURCES FOR POOL DEPLETIONS OF *Saccharomyces cerevisiae* STRAIN K

The cells were grown in dextrose-complete medium and were subjected, after washing, to incubations in nitrogen-free synthetic medium containing either 3 per cent dextrose or 3 per cent ethyl alcohol. Vigorous aeration was maintained throughout the period of starvation. At the ends of the periods noted amino pools were collected and analyzed for the indicated amino acids by means of decarboxylases. The numbers express the results in terms of the percentage of the corresponding non-starved controls.

Exp.	Duration of Starvation	Glutamic Acid		Lysine		Arginine	
		D	Etoh	D	Etoh	D	Etoh
61	70 min.	13	80	33	101	6	112
63	60 min.	27	103	43	97	—	—
	135 min	—	127	—	94	12	189
	180 min.	—	134	—	93	—	—

Here cells were prepared in a manner similar to those described for Table 6, and the resulting suspension was subjected to starvation procedures in the presence of dextrose and ethyl alcohol as energy sources. It is immediately evident, for all three of the amino acids examined, that ethyl alcohol fails to effect a depletion of the free amino acids. On the contrary, one notes a consistent rise above the initial pool levels. Chromatographic analysis of these pools revealed that all components were rising similarly. That the ethyl alcohol does not of itself in some manner prevent the incorporation of the free amino acids into protein, was easily demonstrated by a control experiment in which the ability of glucose to deplete the pool was



examined in the presence and absence of 3 per cent ethyl alcohol. Values of 70 and 68 per cent depletion were obtained. Furthermore, this situation is not confined to alcohol oxidation, as is illustrated by the data in Table 8. Here glucose-grown cells were exposed to their endogenous reserves, glucose and pyruvate, for varying periods of time. Glucose as usual resulted in a marked decrease in pool level. However, in the case of both the endogenous respiration and of pyruvate, one again observes a rise rather than a fall in pool content.

TABLE 8

COMPARISON OF GLUCOSE, ENDOGENOUS SUBSTRATES, AND PYRUVIC ACID AS ENERGY SOURCES FOR POOL DEPLETION

Conditions were similar to those described in Table 7. The numbers express the glutamic acid content of pools expressed as percentages of unstarved controls.

Duration of Starvation (Min.)	Substrate		
	Glucose	Endogenous Substrates	Pyruvate
70	21	135	118
140	21	—	136
210	21.2	165	185

In a sense these results were somewhat paradoxical. The yeast strain used in these experiments can grow in a synthetic medium with either ethyl alcohol or pyruvate as a sole source of carbon and energy. These cells must, therefore, be capable of incorporating amino acids into proteins while metabolizing these compounds. The possibility suggested itself that glucose-grown cells were not adapted to the adequate utilization of these compounds. Consequently, the effect of prior growth on three carbon compounds was examined for their subsequent ability to effect a depletion of the pool. The results obtained are illustrated in Table 9. Cells were grown up in the indicated media and then subjected to "starvation" in the presence of six substrates contained in a nitrogen-free synthetic medium. The extent of the pool depletion was determined by glutamic decarboxylase and is expressed in terms of the percentage of the initial level. Also included for purposes of comparison are the  $Q_{O_2}$  of the cells on the substrates employed. It is evident from Table 9 that glucose



TABLE 9  
THE EFFECT OF PRIOR HISTORY ON THE ABILITY OF VARIOUS CARBON SOURCES TO  
EFFECT DEPLETION OF FREE AMINO ACID POOLS

Cells (Strain K) were grown on the media indicated and were subjected to depletion in nitrogen-free synthetic media with the indicated carbon compound as sole energy source. The numbers represent the glutamic acid content of the free pools as percentages of that found in the unstarved controls. The right-hand side lists the corresponding rates of respiration achieved on the substrates indicated.

Growth Medium	Substrate During Starvation						Q <sub>O<sub>2</sub></sub> on				
	Endogenous	Glucose	Glycerol	Pyruvate	Lactate	Ethyl Alcohol	Endogenous	Glucose	Lactate	Pyruvate	Ethyl Alcohol
Gluc. Compl. (standing)	183	29	167	147	173	—	21	47	28	21	38
Gluc. Compl. (shaking)	118	17	116	161	156	100	20	71	32	28	53
Synth. Pyruv.	73	13	—	85	71	48	—	—	—	—	—
Synth. Lact.	—	—	—	50	46	—	6	100	70	69	88
Synth. Glyc.	—	10	51	—	—	—	9	86	47	63	92

is an effective agent for pool depletion almost independently of the prior history. On the other hand, neither the endogenous substrate nor the three-carbon and two-carbon compounds were effective for glucose-grown cells. Indeed, again considerable rises in pool levels are noted.

However, in the case of cells grown up in pyruvate, lactate, or glycerol, consistent depressions in pool levels are observed as a result of the metabolism of these compounds in the absence of a nitrogen supply. Thus, as in many things, prior history has a profound effect on the response of the free amino acids to a particular energy source.

An examination of the  $Q_{O_2}$  value on the right-hand side of Table 9 would suggest that in part the answer may lie in the rate of energy supply. Thus, in glucose-grown cultures the respiration in the presence of pyruvate is not detectably above that of the endogenous level, whereas in either lactate- or glycerol-grown cells it is considerably elevated, and in such cells pyruvate can effect a pool depletion. However, that this may not be the entire answer is indicated, for example, by the results obtained with ethyl alcohol. Cells grown in dextrose complete medium while being aerated, possess a  $Q_{O_2}$  of 53.5 on ethyl alcohol. Nevertheless, this compound shows no capacity to deplete the pool. Correspondingly, glucose-grown cells which have a  $Q_{O_2}$  of 47 on glucose, suffer relatively severe depletion of their pool in the presence of this compound.

### *C. Ultraviolet activation of the internal replenishing mechanism.*

The intriguing question arises concerning the nature of the labile material which breaks down to replenish the free amino acid pool. An interesting observation, made recently (30) in the course of some experiments with ultraviolet light, may provide an excellent experimental system for a more detailed analysis of this problem. A consistent rise in pool level was observed following ultraviolet irradiation. This rise was subjected to further investigation during the course of a pool starvation. We may briefly, here, summarize the type of experiment which exhibits this phenomenon. Log phase cells of strain K were harvested and washed twice with cold water by centrifugation and suspended in nitrogen-free synthetic medium



containing 3 per cent dextrose. At appropriate intervals samples are removed, centrifuged, and resuspended in water. A portion is employed for an assay of the free amino acid pool in terms of glutamic acid content, and the remainder is treated with ultraviolet. The irradiated cells are centrifuged and resuspended in nitrogen-free synthetic medium containing 3 per cent dextrose and are allowed to incubate, samples being removed for pool determinations. The

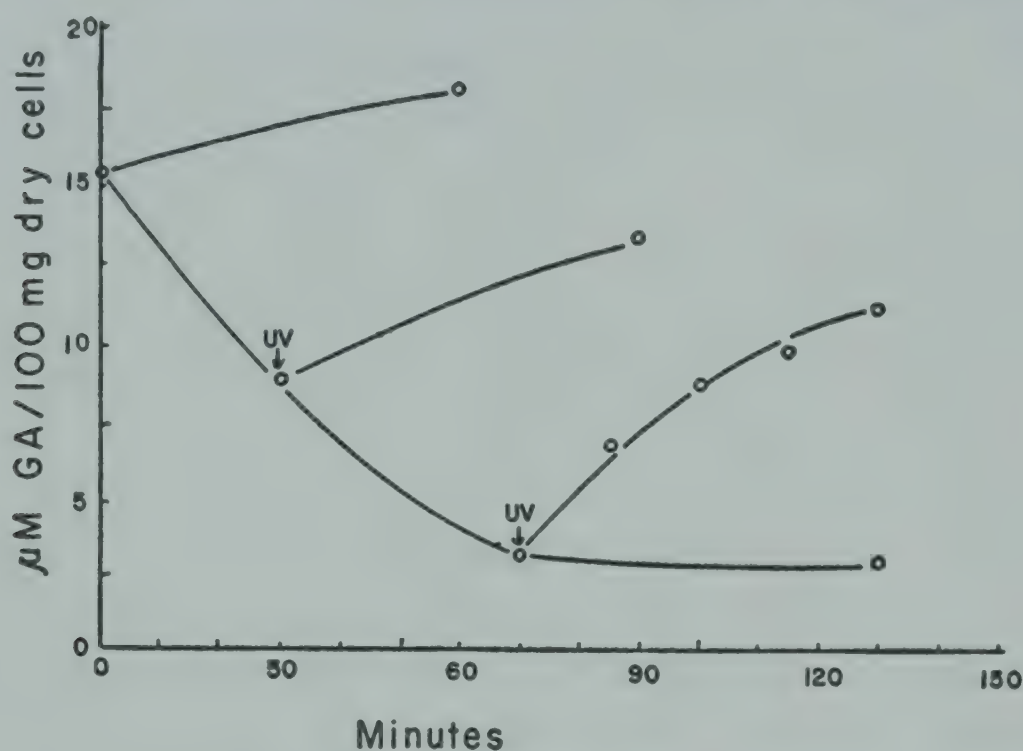


FIG. 1. *The Effect of UV on the internal replenishment mechanism.*

The uninterrupted descending curve describes the normal behavior of the free amino acid pool in the course of a starvation. At zero time and at the times indicated by arrows, aliquots were removed and exposed to UV. The subsequent increase in pool level is described by the ascending curves.

results obtained in such experiments are described in Fig. 1. The normal course of the starvation procedure in terms of the disappearance of glutamic acid is described in terms of the untreated cells. It will be noted that the portions which received UV treatment give the rise in free pool described. The extent of this rise increases in the early stages of the starvation procedure. However, if the starvation is prolonged to extended periods the application of UV results in a lower rise.

The increase in the pool following the ultraviolet treatment during the initial stages of the starvations possesses the same characteristics as those described previously (II, B) for "starvations" carried out

with ineffective carbon sources. As in the latter instance, it has been shown by chromatographic analysis of the pools which accumulate, that the rise following UV treatment involves all the amino acid components. It would then appear that the compounds breaking down are again ones which contain most, if not all, of the amino acids. It seems likely that properly designed experiments with tracers, employing starved and unstarved cells, will lead to the identification of the components involved.

D. *Replenishment of free amino acid pools with individual amino acids.*

We have endeavored to ascertain the extent of the restoration of starved pools which can be achieved by incubating nitrogen-depleted cells with individual amino acids. Expressed as percentages of an  $(\text{NH}_4)_2\text{SO}_4$  replenishment, the results are summarized in Table 10.

TABLE 10  
POOL REPLENISHMENT EMPLOYING INDIVIDUAL AMINO ACIDS  
AS SOURCES OF NITROGEN

12 hr. nitrogen-starved cells were exposed aerobically for 60 min. to a synthetic medium containing 3 per cent glucose and the indicated amino acid at a level of 0.067 M. Pools were then examined for glutamic acid content. The numbers represent percentage replenishment based on that achieved with 1 per cent  $(\text{NH}_4)_2\text{SO}_4$  for the same period of time.

Amino Acid	Percent. Replenishment	Amino Acid	Percent. Replenishment
1. Arginine	46	9. Tryptophan	20
2. Leucine	43	10. Phenylalanine	18
3. Serine	37	11. Tyrosine	18
4. Isoleucine	35	12. Lysine	15
5. Alanine	27	13. Cysteine	12
6. Aspartic	25	14. OH-proline	9
7. Methionine	24	15. Histidine	5
8. Threonine	21	16. Glycine	4

Although some are quite effective, none of the amino acids were capable of full restoration of the pool.

Chromatograms were made of such replenished cells. The amino acid being tested was invariably found in large amounts in the pool.



Ability to restore the glutamic acid content was in general accompanied by a corresponding increase in other components of the pool. Some exceptions were noted. Thus, the 60-minute replenishment with aspartic acid, although it increased the amounts of glutamic acid, alanine, valine, and several others, failed to have an effect upon the arginine and threonine levels. Replenishment with lysine had similar failures with respect to valine, arginine, and isoleucine.

It will be noted that histidine and glycine possess little or no capacity to replenish depleted pools. Rather extensive experiments were undertaken, particularly with glycine, in view of its future use in tracer experiments; and in all cases it was found that the cells were unable to convert glycine into any of the other amino acids. This was further confirmed by experiments in which it was shown that the presence of glycine had no detectable influence on the ease with which a free amino acid pool could be depleted. That we do not have here simply a failure of the glycine to penetrate is demonstrated by the large amounts found in the pools of cells treated with external glycine and glucose.

The procedures described in the preceding sections clearly provide the methods by means of which free amino acid pools can be varied quantitatively as to level or qualitatively with respect to their composition.

#### *E. Implications.*

The results described in the last two sections raise many more problems than they resolve, and it will take a considerable amount of further investigation to clarify completely the relations which exist here. For our present purposes, however, it suffices to emphasize certain implications of these data. In the first place, they illustrate clearly that exposure of cells to a nitrogen-free medium containing an energy source which they can utilize does not *per se* guarantee a disappearance of the internal nitrogen supply. On the contrary, such a treatment may lead rather to the *accumulation* of internal nitrogenous components. This observation is particularly relevant to attempts which seek to extrapolate the results obtained with yeast in one type of experiment to other organisms or to yeast grown



under different conditions. Such phenomena may well explain the rather puzzling results obtained (6, 7) in the adaptation of *Pseudomonas fluorescens* to the utilization of citrate, in which case it is found that the cells adapt more quickly following a "nitrogen starvation" treatment.

A second significant fact which emerges from these experiments is the existence of an internal mechanism which under certain conditions can replenish the free amino acid pool. This is a point of paramount importance for a number of reasons. In the first place, it tells us that the free amino acid pool, as analyzed in a freshly harvested cell, need not, and probably does not, constitute an exhaustive indication of the available internal supply of amino acids in yeast. We shall return to this point, since it is a deduction which can also be arrived at from studying the relation between pool levels and ability to synthesize enzymes. In addition, it should be emphasized that the chromatographic analysis of the rising pools during internal replenishment indicates that the material involved contains all the amino acids. This fact, as well as others which we shall note subsequently, constitutes an extremely important difference in the behavior of yeast as compared with *Escherichia coli*, which possesses no pool and in which no such breakdown of pre-existent protein has as yet been reported.

### III. RELATIONS BETWEEN THE FREE AMINO ACID POOL AND THE SYNTHESIS OF ENZYMES

We now inquire into the role played by the free amino acid pools in the process of the induced synthesis of enzymes. Much of the information obtained on this question and the related one of enzyme precursor in the author's laboratories has dealt with the formation of alpha-glucosidase of yeast, which can split maltose, as well as other alpha-glucosides.

In designing experiments which sought to reveal the nature of the precursor employed by the cell in forming a new enzyme molecule, we were guided by the fact that in principle one can write down three mechanisms of enzyme synthesis:



precursor  $\rightarrow$  enzyme; (1)

precursor + free amino acids  $\rightarrow$  enzyme; (2)

free amino acids  $\rightarrow$  enzyme. (3)

Reaction (1) assumes the preexistence in the non-induced cell of a complex precursor which can be converted into active enzyme without the involvement of the free amino acids. This property distinguishes it from (2) and (3) and permits an experimental decision. It is evident that one plausible approach to the question of deciding between the first and the last two mechanisms is to pose the problem in the form of the following question: Is it possible for the precursor to become active enzyme without the participation of the free amino acid pool? Putting the question this way suggests immediately the necessity for examining the effect on the synthesis of enzyme of any experimental conditions which decrease the availability of the free amino acid pool. There are several methods which were available and have been employed for achieving a restriction of this nature, and they may be listed as follows: (a) the use of amino acid analogues as specific agents to prevent the incorporation of the free amino acids into protein; (b) depletion and restoration of the free amino acid pool under conditions which would minimally disturb other components of the cell; (c) the use of amino-acid-deficient mutants which would make unavailable specific components of the free amino acid pool. Experiments along all of these lines have been realized, with yeast and the bacteria.

In the following sections we summarize, briefly, the available evidence without detailing data which have already been published in extenso.

### 1. *Prevention of free amino acid utilization*

Halvorson and Spiegelman (32) carried out a study with a series of 38 analogues of amino acids for their effect on induced synthesis of alpha-glucosidase in *S. cerevisiae* (strain K). A parallelism was found between the capacity of an analogue to inhibit growth and its ability to suppress enzyme synthesis in non-growing cells. In the case of the effective analogues, complete and specific reversal was



achieved by the addition of the corresponding homologous amino acid. The generality of these findings was extended by the independently performed experiments of Lee and Williams (40a), who demonstrated that the administration of ethionine to the intact rat prevented formation of tryptophan peroxidase.

In the experiments with yeast it was possible to demonstrate that the effective amino acid analogues inhibit net incorporation from the free amino acid pool into the protein fraction. In these studies no evidence for a direct amino-acid-independent transformation of a complex precursor into active enzyme was obtained. The data lead to the conclusion that the primary pathway of induced enzyme formation in non-dividing cells of yeast involves the compulsory utilization of the internal free amino acids. In a more recent study, employing a series of tryptophan analogues, Halvorson, Spiegelman, and Hinman (29a) obtained the same results and arrived at similar conclusions.

One interesting feature which has emerged from these experiments is that the presence of any one effective amino acid analogue can prevent the incorporation, not only of its homologue, but of virtually all the other amino acids as well. The only exceptions observed were serine and glycine. In the case of these two amino acids, nonhomologous analogues did not completely prevent their removal from the free amino acid pool, an observation which is perhaps not surprising in view of the involvement of serine and glycine in the synthesis of heterocyclic compounds.

In summary, the data obtained suggested that the first stable intermediate formed on the way towards the synthesis of an enzyme molecule is of such complexity as to demand the simultaneous participation of a large portion of the various amino acids present.

## 2. *The effect of depletions and replenishments of free amino acid pools on the capacity to synthesize enzymes*

If the conclusions derived from the experiments with amino acid analogues are correct, it would be expected that the ability of cells suspended in a nitrogen-free medium to form enzyme should parallel the levels of the internal pool of free amino acids. Using the methods



described in section II-3A, this question was examined by Halvorson and Spiegelman (33). Employing prolonged (12-hour) nitrogen-starved cells it was possible to modify the pool levels, both quantitatively and qualitatively, with the aid of various replenishment procedures. A strong correlation was established between the enzyme-synthesizing capacity and the degree to which the pool was reestablished to its normal level and composition. The results obtained again support the conclusion that free amino acids constitute the quantitatively predominant source of nitrogen in the formation of new enzyme molecules. No evidence was uncovered which suggested the existence of an amino-acid-independent transformation of a pre-existing complex precursor into active enzyme molecules.

### 3. *The internal replenishing mechanism*

In the course of examining the relation between pool level and enzyme-forming capacity, an interesting inconsistency was observed in the behavior towards starvation of freshly harvested cells, as compared with those which had been subjected to a prolonged starvation and a subsequent replenishment. Thus, it was found that fresh cells starved for 150 minutes suffered a decrease of their pool content of about 80 per cent, which was attended by only a 37 per cent decrease in enzyme-synthesizing capacity. With such cells, the nitrogen starvation had to be continued for 600 minutes to bring the enzyme-synthesizing ability down to about 5 per cent of the unstarved controls. During the period of further starvation, the pool dropped from 20 to 10 per cent of its initial value. This situation is to be compared with prolonged starved cells which have been replenished and then subjected to a second starvation. Here it was found that a 60-minute starvation is sufficient to bring the pool level to 13 per cent of the normal level. At the same time this short starvation virtually abolishes enzyme-synthesizing ability. Evidently in freshly harvested cells enzyme-synthesizing capacity does not for a while parallel the degree of free pool depletion in the course of nitrogen starvation. Parallelism is however rigidly maintained in prolongedly starved cells subjected to replenishment.

At the time these observations were made, we did not have avail-



able the data discussed in sections II-3B and C. It was, however, recognized that the free amino acid pool may not be an exhaustive measure of the available supply of amino acids for the synthesis of new protein molecules. The possibility was proposed that pre-existing protein components might be stimulated to break down to their constituent amino acids, and thus replenish the pool by an internal device. The loss of these labile components upon prolonged starvation could easily explain why such cells, subjected to a short replenishment, are strikingly sensitive to a subsequent starvation. A loss of this kind would be an inevitable consequence of a protracted starvation. Thus, as the starvation process progresses, those proteins which tend to break down easily will do so, and so contribute to the free amino acid pool. Prolongation of the starvation procedure, which continuously recycles material from the pool to the proteins, will tend to trap the amino acids in the least labile proteins, and in the ones which are therefore least capable of supplying free amino acids to the pool. The data obtained from the effects of ultraviolet irradiation, and the attempted starvation of glucose-grown cells with such compounds as ethyl alcohol as energy source, lend strong support to the existence of this sort of mechanism. In so far as the precursor question is concerned, the results show that preexistent complex components can serve as a nitrogen source for enzyme synthesis only by breaking down to their constituent amino acids. In summary, then, the experiments thus far described with yeast lend strong support to the concept that the enzyme-forming mechanism cannot function in the absence of an available supply of free amino acids.

#### 4. *Enzyme formation in amino acid auxotrophic mutants*

The third approach mentioned which could help decide whether a complex precursor can be converted into active enzyme involves the use of auxotrophic mutants deficient in the ability to synthesize one or more amino acids. Experiments started along such lines with yeast quickly ran into complexities not completely unexpected in organisms possessing a large internal pool. No simple relation of enzyme-forming capacity to the external presence or absence of the required amino acid was observed.



This approach was, however, successfully applied almost simultaneously in two laboratories, and it is interesting to note that in both instances the organisms employed, *E. coli* and *Aerobacter aerogenes*, possess no detectable internal supply of free amino acid. One of these investigations stems from the brilliant studies (43) of Monod and Cohn and their collaborators into the formation of beta-galactosidase<sup>1</sup> by the ML strain of *E. coli*. In the course of these studies Cohn and Torriani (15, 16) discovered the existence in non-induced cells of an enzymatically inactive protein (Pz) which was serologically related to the beta-galactosidase. In addition to this obvious structural relationship they established a suggestive correlation between the distribution of the Pz protein and the capacity to synthesize the enzyme. Finally, they showed that a significant decrease in Pz could be exhibited in cells induced to synthesize beta-galactosidase. Although not the only plausible hypothesis considered, it is clear that all of these observations would receive a ready explanation if Pz were the precursor of the beta-galactosidase. In any event, taken together the observations offered the most impressive evidence existent in the literature to support the suggestion that a preexistent complex specific precursor is involved in the synthesis of a known enzyme. The only fact which militated against the acceptance of this view was the observation that nitrogen-starved cells, though possessing normal amounts of Pz, showed no ability to synthesize the enzyme. It was, however, possible to imagine that the starvation procedure interfered in some way with the metabolic step required in the transformation of Pz into active enzyme.

Monod, Pappenheimer, and Cohen-Bazire (45) undertook to investigate this question further by employing a series of mutants, each of which was deficient for a single amino acid. These mutants were subjected to a "specific" starvation by being grown in a medium in which the metabolite required was in limiting quantities, whereas all others were in excess. Immediately upon the cessation of growth as the limiting metabolite became exhausted, an inducer of the beta-galactosidase was introduced. It was found that little or no enzyme was synthesized by cells so treated, despite the fact

<sup>1</sup> Pronounced bā-tə gǎ-lăc-tō-sīd-āse.



that they contained normal amounts of Pz. Such cells do, however, form enzyme immediately upon the addition of the amino acid they require. These results made it necessary to abandon any interpretation of the relation between Pz and the beta-galactosidase which invokes a simple amino-acid-independent transformation of Pz into active enzyme.

Ushiba and Magasanik (66) used essentially the same approach in their study of the adaptive utilization of myo-inositol by amino-acid-, purine-, or pyridine-deficient mutants of *A. aerogenes*. They found that the addition of ammonium sulfate to either the tryptophanless or the leucineless strains had no effect upon the capacity of the cells to form enzyme. However, this type of supplementation greatly augments the enzyme-synthesizing capacity of the wild-type strain. Both mutants were, however, strongly stimulated by the addition of the amino acids which they required. The authors concluded that the induced formation of enzymes involves extensive synthesis from the free amino acids. Subsequently, Rickenberg, Yanofsky, and Bonner (49) reported experiments along similar lines in which beta-galactosidase formation was examined in mutant strains of *E. coli* strain K-12.

### 5. *Is there a precursor?*

The experiments cited thus far would tend to eliminate any mechanism of enzyme synthesis which involves an amino-acid-independent transformation of a preexistent complex precursor into active enzyme. However, none of them eliminate the possibility that a complex precursor is transformed into active enzyme by the addition of free amino acids. Rotman and Spiegelman (52, 53) therefore undertook to provide data relevant to this issue. The most obvious experimental approach aimed at a decision would appear to be the use of isotopic labels. Thus, the induction of enzyme synthesis in uniformly labeled cells suspended in unlabeled medium should provide the necessary data, if the enzyme thus formed can be isolated in a pure state and its isotopic content determined.

It is evident from the type of experiment contemplated that the presence of a large internal free amino acid pool, such as exists in the yeast and many gram-positive bacteria, would introduce com-



plications which could well make the data uninterpretable. Attention was therefore turned to enzyme synthesis in gram-negative organisms, which are known to lack detectable free amino acid pools; and the system selected for study was the beta-galactosidase of *E. coli*.

Uniformly labeled  $C^{14}$ -lactate was employed to grow up labeled cells. Enzyme was induced for short periods of time with the aid of an inducer (melibiose) which does not serve as a carbon source. The beta-galactosidase synthesized was isolated and purified by means of zone ionophoresis through starch columns (39). In some experiments, further purification was achieved with the aid of specific precipitation with purified antiserum (15). The results obtained reveal that less than 1 per cent of the carbon of the newly formed enzyme molecule could have been derived from any cellular components existing prior to the moment of the addition of the inducer. Virtually identical results and conclusions were obtained simultaneously by Cohn and Hogness (see Monod and Cohen, 44, for summary account). These authors employed  $S^{35}$  as the isotopic label and primarily immunological procedures for the purification of the enzyme.

These findings virtually eliminate any hypothesis which assumes the preexistence of a precursor material, simple or complex, which is convertible into enzyme. It is evident that the third mechanism, suggesting formation of enzyme *de novo* from free amino acids, is at present the only one which has received experimental support.

#### 6. Protein synthesis in *E. coli* and *S. cerevisiae*

Experiments with yeast and *E. coli* have provided identical answers with respect to the question of the precursor employed in enzyme synthesis. Both types of material have emphasized the importance of free amino acids and have yielded data difficult to interpret except in terms of synthesis *de novo*.

While there are great similarities in the conclusions derivable from experiments with *E. coli* and yeast, there are, nevertheless, differences in detail which it seems worthwhile to emphasize explicitly. It is evident, for example, that the tracer experiments with



*E. coli* would have failed had there been any extensive turnover of the proteins in the growing cells. All the proteins existing prior to the moment of induction were heavily and uniformly labeled. Had they broken down to their constituent amino acids, these would have appeared in the beta-galactosidase synthesized, even if the process of enzyme formation does not involve any preexistent complex precursor. The fact that no detectable label was found in the beta-galactosidase formed suggests that for all intents and purposes, protein synthesis in growing cells of *E. coli* is virtually irreversible. Cohn and Hogness (44) have attempted to determine turnover in the beta-galactosidase itself in the presence and absence of inducer, and failed to find any. It should be emphasized that these results do not mean that the proteins of *E. coli* are inordinately less labile than those of, for example, the mammalian liver. The experiments performed would just barely have detected a half-life of  $6\frac{1}{2}$  days. Nevertheless, it should be noted that a turnover in *E. coli* even remotely approximating its synthetic rate (ca. 1,000-fold greater than that observed in adult liver) was not detected.

It is evident that the situation just described in *E. coli* does not obtain in yeast. Here, one can provide convincing evidence that there do exist components in the cell which can break down into their constituent amino acids. This difference immediately raises another issue which is directly pertinent to the question of the interaction between enzyme-forming systems, and the level at which interaction takes place.

It has been demonstrated by a number of workers (43, 49) that removal of the inducer does not lead to the destructive disappearances of the beta-galactosidase in *E. coli*, but rather to its dilution as the cells grow. This situation is quite unlike that observed in the case of inducible enzymes studied in yeast. Here, removal of inducer and a supply of an energy source leads to the disappearance of the induced enzyme (58). It is interesting and provocative to note that this disappearance requires a functional energy supply. Thus, any agent which uncouples the energy-generating mechanism, leads to stabilization of the enzyme in the absence of its inducer. Further experiments with suitably labeled induced cells should provide



information which could help establish the nature of this interaction.

The differences between the behavior of the beta-galactosidase of *E. coli* and the alpha-glucosidase of *S. cerevisiae* should make one cautious about the desirability of studying a variety of enzyme systems in a diversity of organisms if an adequate account of the properties of enzyme- and protein-synthesizing systems is to be attained. The brilliance of the experiments accomplished with the beta-galactosidase of *E. coli* should not blind us to the obvious fact that it is after all only one enzyme system. It need not necessarily be the prophetic symbol which will lead us inevitably to the complete knowledge of the synthesis of all enzymes in all organisms, or even of all enzymes in *E. coli*.

Thus, the remarkable stability of beta-galactosidase in the absence of inducer is evidently not shared by all inducible enzymes of *E. coli*. Fowler (20) has described a situation involving the stability of a fermentation enzyme complex which, in its instability and relation to an energy supply, is virtually identical to that found for the alpha-glucosidase of yeast. Similarly, Sher and Mallette (54) have demonstrated that the inducible lysine decarboxylase of *E. coli* suffers destructive disappearance in the absence of its inducer.

#### IV. THE NATURE OF THE ENZYME-FORMING SYSTEM

In the present discussion the term "enzyme-forming system" (hereinafter referred to as EFS) will be used to designate that structure in the cell which is *directly* involved in the process of fabricating the enzyme molecule. This device is employed to isolate EFS conceptually from all the other cellular components which can, and probably do, intervene more or less indirectly. It will of course be noted that there is an assumption involved here, for by so stating the problem we do presume the existence of such a unique structure and at least implicitly ignore the possibility that proteins and enzymes are formed by a multitude of cooperating and sequential reactions.

##### *A. Implications of the Precursor Findings for the Nature of the EFS.*

In thinking about the possible nature of the EFS, and in designing experiments to identify its chemical properties we were understand-



ably influenced by the results of the investigations into the precursor question. In a sense, these findings forced us to begin the search for the EFS. The data we have reviewed in previous paragraphs on the question of enzyme and protein precursors are satisfyingly clearcut, almost distressingly so. They lead compellingly to the conclusion, that in fabricating a new enzyme molecule, the cell prefers to weave it rather than to stamp it into existence. In this process the simplest components (amino acids) are employed. Further, we find no evidence for any stable intermediate smaller than that requiring the simultaneous presence and utilization of all the amino acids. Indeed, the available information leaves one with serious doubts that there is any recoverable intermediate between the free amino acids and the finished product. It looks rather as if at one moment we have free amino acids and the next an enzyme molecule. From one point of view this is, of course, a pessimistic conclusion. It suggests that we will not achieve a successive approximation to an understanding of how enzymes are synthesized in terms of a gradually better insight gleaned from the study of intermediate pieces of increasing complexity, as they approach the final stage of synthesis. A door is thus slammed upon an extremely attractive approach for tackling the question. It need hardly be added explicitly that this view is not yet a logically necessary deduction from the available evidence, and it is to be hoped that not everyone will accept it. Those, however, who feel compelled to do so are faced with the necessity of finding a new pathway to the solution of the problem of protein synthesis.

The acceptance of a mechanism of protein synthesis which involves the simultaneous utilization of the constituent amino acids leads one quite naturally to consider a template-type mechanism. We cannot here undertake an extensive discussion of the recent information obtained from radioactive experiments with intact animals and tissues which have attempted to decide between the template and stepwise mechanisms (2, 3, 4, 10, 11, 56). In general, unequal labeling of a protein has been taken as an argument against the simultaneous utilization of a common free amino acid pool. That this is not a necessary deduction from such data has been pointed out by Dalglish (18).



A more serious difficulty is introduced by the realization that the incorporation of labeled compounds need not provide us with information solely on the question of total protein synthesis, since exchange reactions are necessarily included in the information obtained from such experiments. That a very real difficulty exists is well illustrated by the beautiful experiments recently reported by Gale and Folkes (27, 28). In studying the incorporation of labeled glutamic acid into the proteins of *Staphylococcus aureus*, these authors have exhibited the possibility of dissociating net protein synthesis from incorporation by exchange reactions. As had been shown with yeast (32), these authors find that *p*-chlorophenylalanine can prevent new protein synthesis in *S. aureus*. In addition, it is revealed that although the presence of this analogue effectively prevents the incorporation of phenylalanine into protein, it has relatively little effect on the exchange incorporation of glutamic acid. Convincing evidence has been provided that the glutamic acid incorporated under this condition has indeed become a part of the protein molecules of the cell.

The existence of such phenomena raises the question whether incorporation experiments which yield data indicating uneven labeling of a particular amino acid along a protein molecule may not be telling us something about the geometry of the protein molecule, in terms of ease of exchange reactions, rather than about the mechanism employed in putting this molecule together.

As soon as one makes a template assumption it is quite evident that in structure the template must be at least as complicated and as large as the molecule which it is forming. With this assumption there are relatively few known candidates that one can propose which could satisfy the two criteria of relatively large size and complexity. Large size alone is clearly not sufficient to effect the synthesis of substances like protein with their wide range of biological specificities. With these restrictions in mind, the three known candidates one can propose are desoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. It should be noted that by putting the matter this way we do not preclude the possibility of complexes between these kinds of compounds being the actual functioning EFS.



In the following paragraphs we should like to examine the available evidence for and against the possibility that one or more of these substances is associated with the enzyme-forming system. Since the system being studied necessarily involves protein synthesis, it is extremely difficult to design adequate experiments which would properly test the possibility that the EFS is protein in nature or contains protein as a component.

### 1. *The Unlikelihood of DNA as EFS*

Definitive evidence eliminating the personal involvement of DNA in the synthesis of protein has thus far not been provided. Presumably, an unequivocal demonstration, one way or the other, will ultimately come from experiments analogous to those with enucleated fragments of protozoa. Brachet and Chantrenne (9) have shown that such fragments incorporate label into proteins as readily as their nucleated counterparts. The possibility that this may represent an exchange reaction, rather than the synthesis of new protein molecules, make conclusions from such data uncertain.

There exists a variety of experiments indicating the possibility of dissociating DNA from protein synthesis. DNA synthesis is known (1) to be far more sensitive to inhibition by irradiation with x-rays than is protein formation. Baron, Spiegelman, and Quastler (5) have shown that x-ray dosages (350,000r) far exceeding those expected to stop the formation of DNA completely, permit normal enzyme synthesis in yeast.

Sher and Malette (54) found that cells of *E. coli* treated with sulfur mustard form lysine decarboxylase at rates comparable to untreated controls. Herriot (35) had previously shown that sulfur mustard, at the concentrations employed, completely abolished DNA synthesis.

Kelner's (37) studies on photoreactivation of bacterial cells after exposure to UV have provided an elegant method for a virtually complete separation of RNA formation and cell growth from the net synthesis of DNA in *E. coli*. Halvorson and Jackson (31), using yeast, have recently repeated and confirmed these results in following the synthesis of maltase, protein, DNA, and RNA. The



results obtained suggested that protein and RNA continue to be synthesized at UV dosages which inhibit DNA formation. A similar situation has been achieved in preliminary experiments (29) by the use of proflavine on *E. coli*.

Cohen and Barner (17) have recently reported the ability of a thymineless mutant of *E. coli* which can produce xylose isomerase in the absence of added thymine. Dr. Cohen was kind enough to send us a duplicate of this mutant, and we have succeeded (8a) in confirming this finding in the case of the induced synthesis of beta-galactosidase. Washed cells of this strain, when suspended in synthetic medium lacking thymine, synthesize considerable amounts of enzyme in the presence of a suitable inducer. This behavior is in striking contrast to that observed for other metabolic deficiencies. Thus, in our own experience and in that of others (45), uracil-deficient, adenine-deficient, or amino-acid-deficient mutants treated in the same manner form little or no enzyme in the absence of the required metabolite.

The data cited above demonstrate that drastic interference with DNA synthesis is often not accompanied by very striking effects on the formation of protein. While this does not eliminate DNA as a component of the EFS, it hardly lends support to the supposition that it is such. In any case, we have not been encouraged to design further experiments with this as a working hypothesis.

## 2. Evidence for RNA as an EFS Component

Many have postulated that RNA is a key substance in protein synthesis. Chantrenne (13) has succinctly summarized such concepts and the evidence which supports them. Certainly recently, the most compelling data have emerged from the exciting experiments of Gale and his collaborators (23, 24, 25, 26, 27, 28). Here striking correlations have been established between protein-synthesizing capacity and nucleic acid metabolism. Since these studies will undoubtedly be discussed by Dr. Gale at this symposium we reluctantly forego the pleasures of a Cicero in guiding the audience through the beautifully clear-cut results obtained.

We will confine our attention primarily to some of our own results



which confirm, with the system we have studied, many of the conclusions derivable from the data obtained by Gale with *S. aureus*.

#### *A. Experiments with Ultraviolet Light.*

Swenson and Giese (62a) have demonstrated that at sufficiently elevated doses, UV can inhibit the induced synthesis of enzymes in yeast. The dosages required are far above those found (31) to be adequate for the dissociation of DNA from RNA and protein synthesis. An examination (62) of the action spectrum of the inhibition of enzyme formation revealed that it coincided with the adsorption spectrum of nucleic acid.

Halvorson and Jackson (30) examined this system further in an attempt to see whether the dosages required to stop enzyme synthesis corresponded to those which inhibit utilization of the free amino acid pool in yeast. The experiments were carried out with washed exponential-phase cells of strain K, precautions being taken to avoid photoreactivation. Subsequent to irradiation, ability to incorporate amino acids from the pools was tested by an 80-minute aerobic incubation at 30° C. in nitrogen-free synthetic medium (32) containing 3 per cent glucose. Pools were examined for glutamic acid and arginine content by the decarboxylase procedure. Capacity to form enzyme was analyzed by an 80-minute aerobic induction with cells suspended in nitrogen-free synthetic medium containing 3 per cent maltose. Following the induction, the cells were centrifuged, washed and "fast dried." Their alpha-glucosidase content was examined by their ability to split alpha-phenyl-D-glucoside (Spiegelman, 61). The results obtained are summarized in Fig. 2. Excellent parallelism is observed in the loss of the capacities to incorporate amino acids into protein and to synthesize the alpha-glucosidase. A dosage of 80 seconds or more is sufficient here virtually to abolish the synthesis of new protein molecules.

It is of interest to note that pool replenishment is far more resistant to UV irradiation. This is demonstrated by the results shown in Fig. 3. Here pool-depleted cells were subjected to UV-irradiation for the periods indicated. They were then allowed to replenish their pools by exposure to 1 per cent  $(\text{NH}_4)_2\text{SO}_4$  and 3



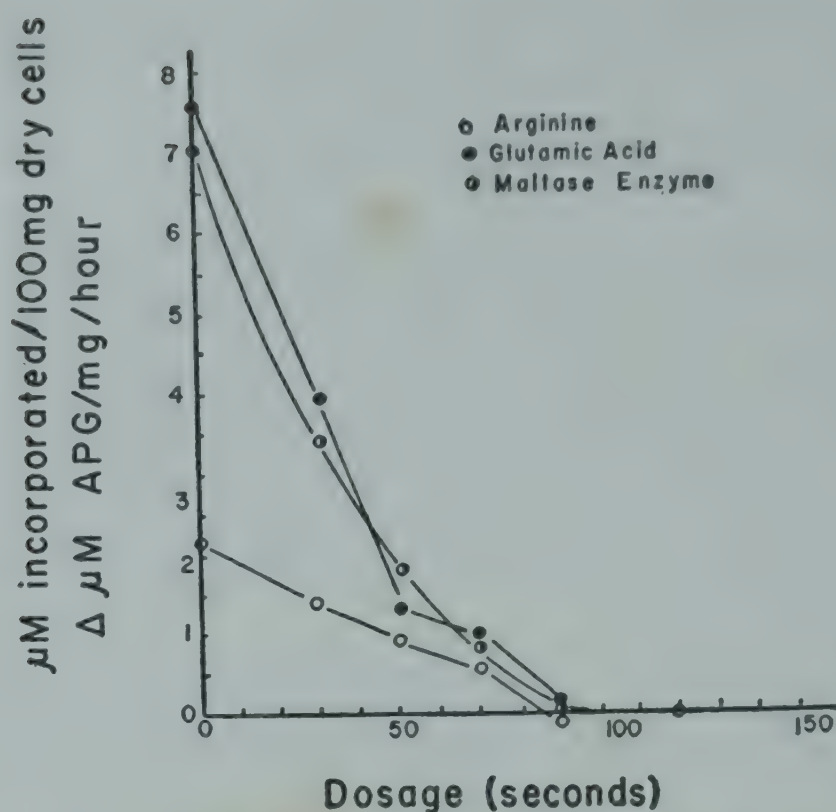


FIG. 2. The effect of UV on utilization of the free amino acid pool and enzyme (*alpha*-glucosidase) synthesis in yeast.

The arginine and glutamic acid contents were determined by decarboxylases. Enzyme was assayed in terms of the rate of splitting of *alpha*-phenyl-D-glucoside (APG).

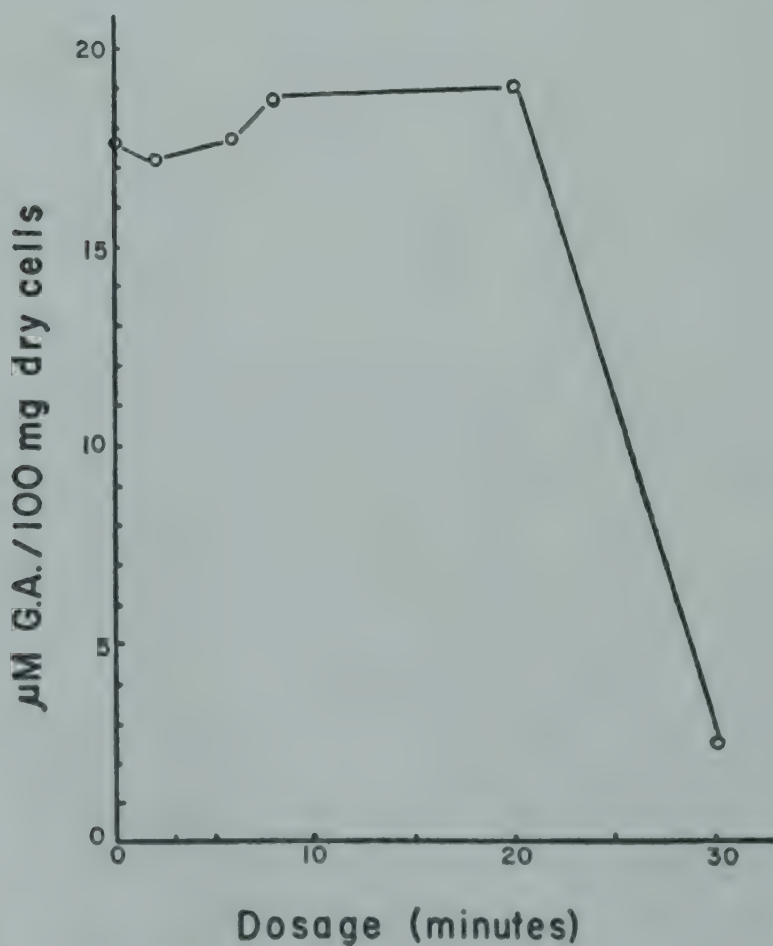


FIG. 3. The effect of UV on replenishment of the free amino acid pool in starved cells.

per cent glucose for 60 minutes. The degree of replenishment was ascertained by an analysis for glutamic acid. It is evident that dosages below 1200 seconds have little or no effect on the replenishment process.

An analysis has been instituted of the effects of ultraviolet irradiation on the ability of RNA to incorporate  $P^{32}$ , in an attempt to see whether dosages known to inhibit protein synthesis influence RNA metabolism. A sample experiment may be briefly described. Subsequent to UV-irradiation log phase cells (Strain K) were induced to form alpha-glucosidase for 80 minutes in the presence of  $P^{32}$  and 3 per cent maltose. Aliquots were removed for enzyme assay, and the remainder was centrifuged and washed three times with cold  $H_2O$ . Nucleotides, prepared according to the procedures of Juni et al. (36), were separated on resin columns (14). The results, shown in Table 11, indicate that rather severe effects on enzyme

TABLE 11

THE EFFECT OF UV IRRADIATION ON ENZYME SYNTHESIS AND  $P^{32}$  INCORPORATION INTO RNA

UV dose (seconds)	Enzyme Synthesized	Per cent of Control	$\gamma$ RNA/ml.	cts./min./ $\gamma$ P (cytidylic)	Per cent of Control
0	59	100	3.10	314	100
30	35	59	2.92	277	88
70	3	5	2.92	244	78
120	0	0	3.06	150	48

synthesis are achieved at dosages which have relatively little effects on RNA metabolism. Even at the 120 sec. dose, which completely abolished enzyme synthesis and net utilization of the free amino acid pool, 48 per cent of normal incorporation into RNA is still observed. Similar results are obtained on examination of the adenylic guanylic, and uridylic acids. The data suggest that if RNA is directly involved, even slight damage to its metabolism can have profound effects on protein-synthesizing capacity.



*B. The Effect of Purine and Pyrimidine Analogues on Enzyme Synthesis.*

One obvious approach which could in principle yield information pertinent to the role of nucleic acid is to examine the effect of various purine and pyrimidine analogues on the process of enzyme synthesis. A study of this kind has been undertaken in our laboratory (8a). We will not, here, undertake to describe all the experiments performed but will confine ourselves to a few representative observations.

The system studied was the formation of beta-galactosidase in a purineless mutant of *E. coli* (Strain M-55B-46). Cells were grown in Davis synthetic medium (19) supplemented with 50 $\gamma$  per ml. of adenine and containing 2 per cent glycerol as the energy source. Enzyme was assayed subsequent to cell lysis with toluene by means of the chromogenic substrate, orthonitrophenyl-beta-D-galactoside (ONPG), according to the procedure devised by Lederberg (40). It is well known (43) that enzyme synthesis in the *E. coli* organism is quite rigidly linked to growth. Thus, any agent or condition which prevents growth of the cells inhibits enzyme formation. Conclusions from experiments in which growth has been prevented are therefore uncertain, since the absence of enzyme formation in such a situation may be a secondary consequence of the growth cessation. It is necessary, therefore, in employing this system to attempt to achieve conditions of treatment, or concentration of agents being tested, which do permit cell increase.

Monod and his collaborators (45) have suggested a very convenient method for examining the *relative* rate of enzyme formation in a manner which automatically takes into account any effects on the overall growth rate of the cell. The procedure consists in plotting increase in enzyme activity against increase in mass of the cells. One may thus study fast- or slow-growing cultures and obtain from the slope of such a plot the relative rate of enzyme synthesis independently of the actual growth rate.

The data of a typical experiment with 5-OH-uridine are described in Fig. 4. It will be noted from the right-hand side that the amount of the uridine derivative employed (5 $\gamma$  per ml.) had no detectable



influence upon the growth rate of the cells. A dramatic effect is, however, observed on the ability of these cells to form beta-galactosidase. There is a complete inhibition of enzyme formation when the compound is introduced simultaneously with the inducer (n-butyl-beta-D-galactoside). After a while the cells recover from this

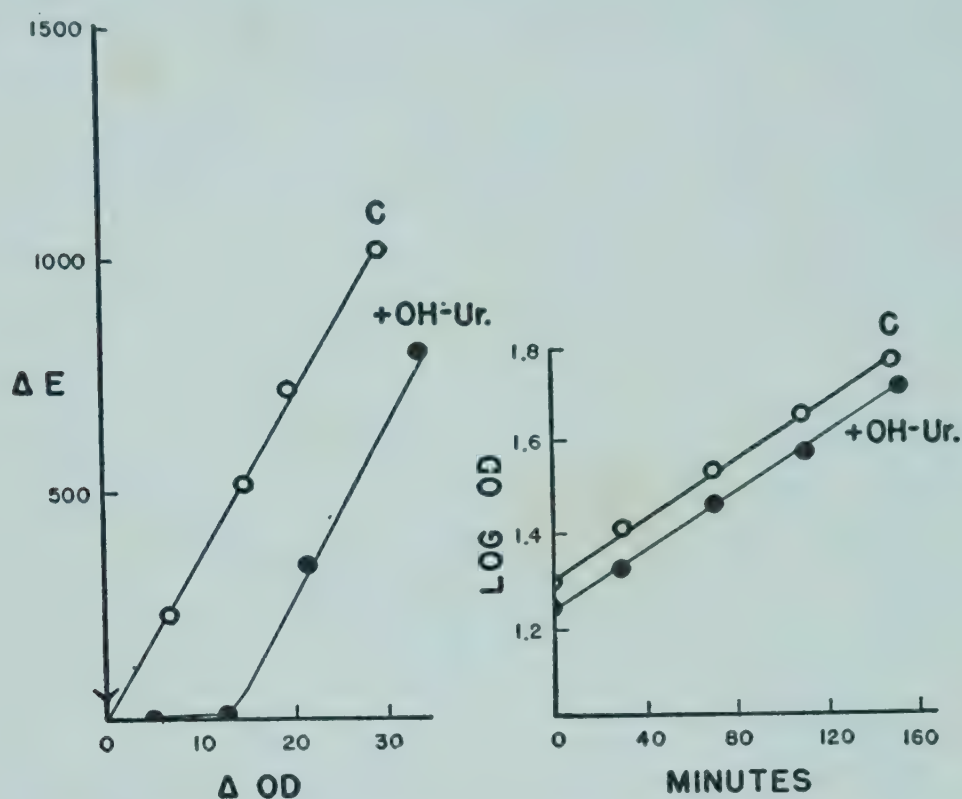


FIG. 4. The effect of 5-OH-uridine on the induced synthesis of beta-galactosidase in *E. coli*.

On the left,  $\Delta E$  denotes increments in enzyme in terms of the rates of hydrolysis of ortho-nitrophenyl-beta-D-galactoside (ONPG) per ml. per unit time, measured on toluem lysates.  $\Delta(OD)$  measures change in density measured with a Klett-Sumerson colorimeter. Curves marked C, control systems. Inducer and 5-OH-uridine were introduced simultaneously at zero time in the experimental curve (indicated as +OH-Ur.).

inhibition. This escape is very likely due, as suggested by Slotnick et al. (57), to metabolism of the OH-uridine by the cells. This suggestion was substantiated by experiments in which small amounts of the OH-uridine were continuously added. Under such conditions the cells did not escape the inhibition.

It was of interest to see whether this compound could stop enzyme synthesis once the latter has been instituted. Fig. 5 summarizes the results of this type of experiment, using the same strain. It is evident that even with the enzyme synthesis actively in progress the OH-uridine can effect an immediate cessation.

These experiments are illuminating from several points of view.



In the first place, it is evident that at the concentration employed, the OH-uridine is not acting as a general inhibitor of protein synthesis. Nevertheless, there is observed a rather complete cessation of the formation of a particular protein. The experiments of Roberts and Visser (50) would suggest that OH-uridine prevents the utilization of uracil for the synthesis of RNA. The apparent specificity

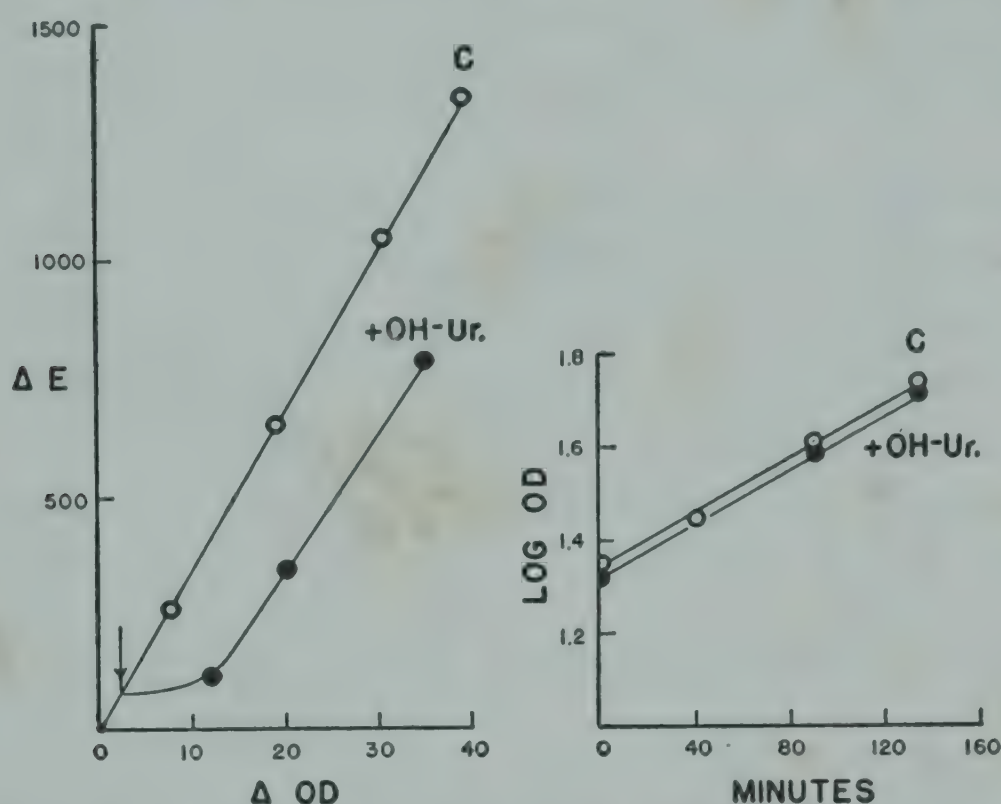


FIG. 5. The effect of 5-OH-uridine on the synthesis of beta-galactosidase subsequent to the onset of induction.

Ordinates and abscissae are the same as in Fig. 4. Inducer was introduced at zero time in both experimental and control. 5-OH-uridine was introduced at the point indicated by the arrow.

of the inhibition in terms of the synthesis of beta-galactosidase can be explained if it is assumed that the beta-galactosidase enzyme-forming mechanism is a relatively poor competitor for RNA precursors. Hence, under conditions in which competition is made severe by limitation, this enzyme-forming system suffers relative to the other protein-synthesizing systems of the cell.

Secondly, the experiment described in Fig. 5 suggests that continued synthesis of RNA is required for the uninterrupted production of enzyme. Were it possible for the enzyme-forming units to function without new RNA formation, the addition of the OH-uridine should have led to a possibly lower rate of enzyme formation rather than have resulted in an immediate cessation. Further evidence for this conclusion will be discussed below.



*C. The Effect of Amino Acids and Purines and Pyrimidines on Enzyme Synthesis in E. coli.*

The concept of competition between enzyme-forming systems for some RNA precursor suggested an explanation for a curious phenomenon we had observed earlier. In the course of studying the factors which could increase the ability of *E. coli* cells to synthesize beta-galactosidase, the effect of an externally added amino acid mixture was examined. It was found, quite surprisingly, that far from aiding the formation of this enzyme, the addition of amino acid mixtures to a minimal medium led to an immediate cessation of beta-galactosidase synthesis. At the same time the growth of the cells was increased.

One could explain the inhibitory effect of the amino acids on beta-galactosidase synthesis in terms of the following chain of events. The ready availability of preformed amino acids resulted in a sudden stimulation of the protein-synthesizing systems, as evidenced by the increased growth rate. This in turn might well lead to an exhaustive demand on the metabolic devices which supply the derivatives involved in nucleic acid synthesis. Under such conditions any system which was a poor competitor for RNA precursors might well be suppressed even though, and indeed precisely because, total synthesis of protein was being accelerated. Hence, the beta-galactosidase-forming system was suppressed.

This possibility was tested (29) by an attempt to increase the available supply of RNA precursors. This was done by incubating cells for thirty minutes prior to induction in a lactate synthetic medium supplemented with uracil, adenine, guanine, and cytosine all in equimolar amounts at a level of  $0.8 \mu\text{M}$ . per ml. The effect of this prior incubation on the subsequent behavior towards the presence of external amino acids (at a level of 0.1 per cent of a tryptic casein digest) is seen in Fig. 6. The control suspension experienced the usual severe inhibition of beta-galactosidase formation upon the addition of the amino acid mixture with the inducer. The inhibition period extends in time to about 40 minutes. It will be further noted that the preincubation completely abolishes the inhibition. It seems likely that the recovery invariably observed in



the control cells is due to an internal adjustment of the nucleotide metabolism in the presence of a ready supply of amino acids. This matter is being further investigated.

Such observations emphasize the importance of purine and pyrimidine metabolism not only in overall protein synthesis but also in the more subtle kinds of interactions which can occur amongst

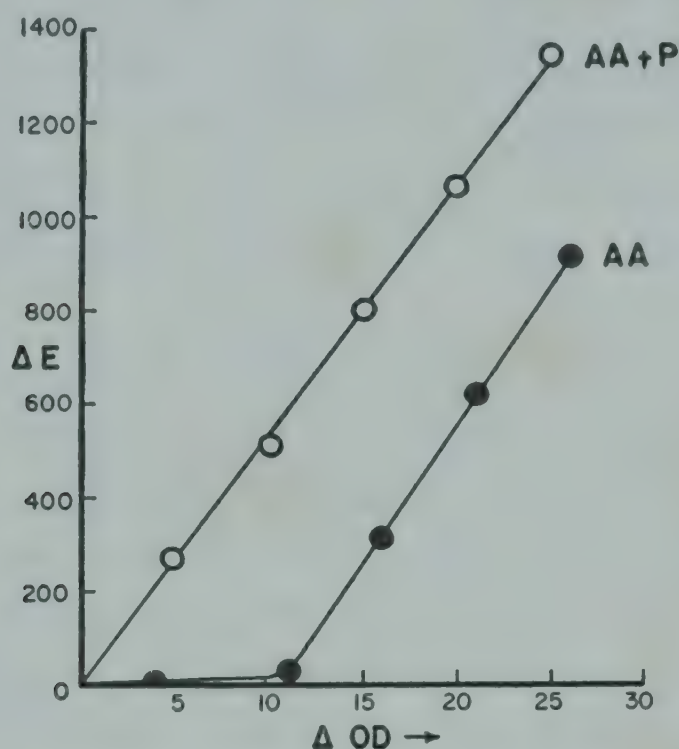


FIG. 6. The effect of amino acids on enzyme synthesis with and without prior incubation with purines and pyrimidines.

Ordinate and abscissa are same as in the left-hand side of Fig. 4. Inducer and amino acid mixture were introduced at zero time. Cells described by curve (AA+P) were exposed to a purine and pyrimidine mixture for 30 min. prior to zero time.

protein-synthesizing systems. Such interactions can only be exhibited and studied in experiments which examine the synthesis of specific proteins.

Some puzzling observations on the induced synthesis of various enzymes can be readily interpreted in terms of these interactions. Thus, the formation of hydrogenlyase in *E. coli* (8, 47) will occur during growth in a synthetic medium only if it is supplemented with amino acids. This suggests that the enzyme-forming mechanism involved is a good competitor for RNA precursors, and a relatively poor one for amino acids. The addition of OH-uridine might well stimulate this system rather than suppress it. The same view could also explain why the aging of cultures can result in the more rapid synthesis of this enzyme.



*D. Behavior of Purineless and Pyrimidineless Mutants of E. coli.*

One of the characteristic features of the induced synthesis of beta-galactosidase in *E. coli* is that enzyme formation ensues at the maximal relative rate immediately upon the introduction of the inducing agent. This would imply that normally cells contain the corresponding enzyme-forming system ready to function when exposed to the proper inducing agent.

Unless the EFS involved is performing another necessary function, its presence would be completely dispensable for cells growing in a medium containing carbon sources other than lactose. If the EFS does contain RNA, one might expect to force the cell to dispense with its synthesis by growing them under conditions which impose severe limitations on RNA precursors. Successful suppression of the formation of the beta-galactosidase-forming system by such treatments should be reflected in a lag in the onset of beta-galactosidase synthesis on introducing the inducer. Experiments attempting to accomplish this were performed with the aid of purineless and pyrimidineless strains of *E. coli*. These were grown under conditions which subjected them to various degrees of severity of limitation for the metabolite they required. The characteristics of the beta-galactosidase synthesis were then examined in the presence of an excess of the needed metabolite.

In performing such experiments it is of course important not to subject the cells to such severe conditions of limitation that they completely exhaust themselves of the compound and its derivatives. Such exhaustive depletions can, and do, result in a generalized and widespread interference with metabolism. This is a caution particularly applicable to mutants possessing adenine and uracil deficiencies. "Limited" depletions which possess no detectable effects on overall metabolism or synthetic capacity can be achieved. This may be illustrated by a representative experiment with a uracilless strain of *E. coli* (M-63-86). This strain was grown in a medium containing limiting amounts (1 $\gamma$ /ml.) of uracil. Immediately preceding its entrance into the stationary phase, it was diluted into a synthetic medium containing excess uracil and inducer. A companion control culture was grown in excess (100 $\gamma$ /ml.) uracil and was diluted to



the same optical density into the identical medium. The beta-galactosidase formation was followed in the usual way. The results obtained are shown in Fig. 7. It will be noted that the two cultures were physiologically indistinguishable in so far as their growth rates are concerned. Nevertheless, the culture which had been subjected to the limiting growth in uracil exhibits a marked lag in beta-galactosidase synthesis. On the other hand, the control culture begins to

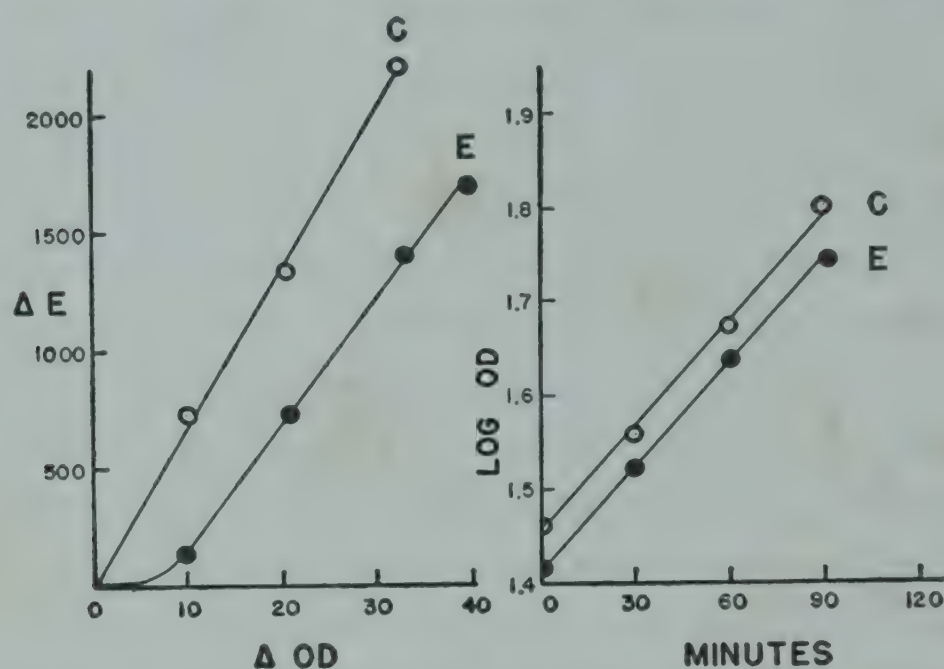


FIG. 7. The effect of growth in limiting uracil on subsequent ability to synthesize beta-galactosidase.

Ordinates and abscissae are the same as in Fig. 4. Cells of curve C were grown in excess uracil, those of curve E were grown in limiting uracil. Inducer and excess uracil added at zero time in both cases.

form enzyme at the maximal rate immediately. Analogous observations have been made with adenine mutants.

Such findings take on added significance in view of the fact that one does not in general observe such "specific" inhibitory effects on beta-galactosidase synthesis in cells similarly treated but having a limited availability of other metabolites (e. g., amino acids, carbon and energy supply, sulfur, 45, 8a).

It would appear that growth under conditions which make RNA synthesis difficult leads to the loss or suppression of the beta-galactosidase-forming mechanism. This loss is, however, easily restored by exposure to the required purine or pyrimidine. The results obtained are predictable from the assumption that RNA is part of the EFS.



*E. The Behavior of Pyrimidineless and Purineless Mutants of Yeast.*

As is our practice, we paralleled our studies of *E. coli* with a comparable examination of yeast strains (6a). We immediately came upon observations which were in apparent contradiction to the concept that RNA was a controlling substance in enzyme synthesis. Thus, purine and pyrimidine analogues which were effective antagonists in the *E. coli* system exerted no detectable ability to inhibit alpha-glucosidase synthesis in homologous yeast mutants. Again, unlike the observation with *E. coli*, purineless and pyrimidineless mutants of yeast synthesized enzyme as readily in the absence of the metabolite they required as they did in its presence. Finally, the enzyme-forming capacities of these mutant types were not markedly different from those of their wild type counterparts.

In view of our experience with the free amino acid pool, it was considered possible that the apparent relative independence of the enzyme-forming mechanism from external supplements might find its explanation in terms of the existence of an internal supply. This was very quickly found to be the case. Employing cold TCA extraction (38), the existence of an acid-soluble pool of purine and pyrimidine derivatives was established (8a). The components were examined for by chromatographic procedures (12, 68, 67, 41), both qualitative and quantitative. Our analysis as to all components is not yet complete. However, we can state at the present time that quantitatively the major components of the uracil mutant's pool are adenylic acid, guanylic acid, and uridine. No free bases, other than the ones used as supplements in the medium, are found in the free pool.

With the existence of a nucleotide pool established, it became necessary to devise procedures to control its level, analogous to what has been accomplished with the free amino acid pool.

It was found that the procedure employed in depleting the free amino acid pool was only slightly effective in removing the free nucleotide pool. A successful method was evolved, based on the assumption that the nucleotide pool was more actively used during rapid protein synthesis. It was reasoned that the inability to deplete the nucleotide pool by the ordinary procedure of incubating in a



nitrogen-free medium containing glucose stemmed from the fact that the free amino acid pool was relatively quickly exhausted, leaving behind a still unutilized portion of the nucleotide pool.

It seemed probable that this difficulty could be obviated by including a mixture of amino acids in the depletion treatment. This procedure met with immediate success. Thus, aerobic incubation of a pyrimidineless mutant for one or two hours in a synthetic medium supplemented with amino acids (0.4 per cent casein hydrolysate) and 6 per cent glucose, leads to the virtual elimination of pyrimidine derivatives from the free pool. Such treatments tend to increase the guanylic acid levels but do not modify the concentrations of adenylic acid.

It was found easily possible to replenish such depleted pools by incubating the cells aerobically in the presence of glucose in a mixture of purine and pyrimidine bases (uracil 30 $\gamma$ /ml.; cytosine 60 $\gamma$ /ml.; guanine 100 $\gamma$ /ml.; adenine 90 $\gamma$ /ml.). Actually, the presence of guanine and adenine were not necessary for replenishment of the uracilless mutant. The details of the behavior of these pools in various strains will be reserved for subsequent publication. It may, however, be remarked here that depletion by such procedures can be effected even in wild-type strains. The indication is that in yeast, as in *E. coli*, forcing rapid protein synthesis can temporarily lead to an exhaustion of nucleotide components.

It should be noted that all the operations leading both to depletions and to replenishments of the nucleotide pool were carried out in the presence of a continuous external supply of free amino acids. The free amino acid pools of such cells were, as a consequence, left undisturbed.

Having acquired specific control over the levels of the nucleotide pool, it became possible to perform properly designed experiments which inquired into the role the nucleotides played in enzyme synthesis. It was found that the ability to synthesize alpha-glucosidase paralleled nucleotide pool levels both in depletion and replenishment experiments. Fig. 8 exhibits a typical set of results obtained with a uracilless mutant subjected to the procedures described above.

Complete replenishment of the nucleotide pool by means of a 60-minute incubation in the purine-pyrimidine mixture results in a

dramatic restoration of enzyme-forming capacity (Curve R-60') to a level exceeding that of the untreated controls. Partial replenishment (Curve R-20') leads to partial restoration. The delayed, limited recovery exhibited by the depleted cells (Curve D) may be a reflection of an internal replenishing mechanism.

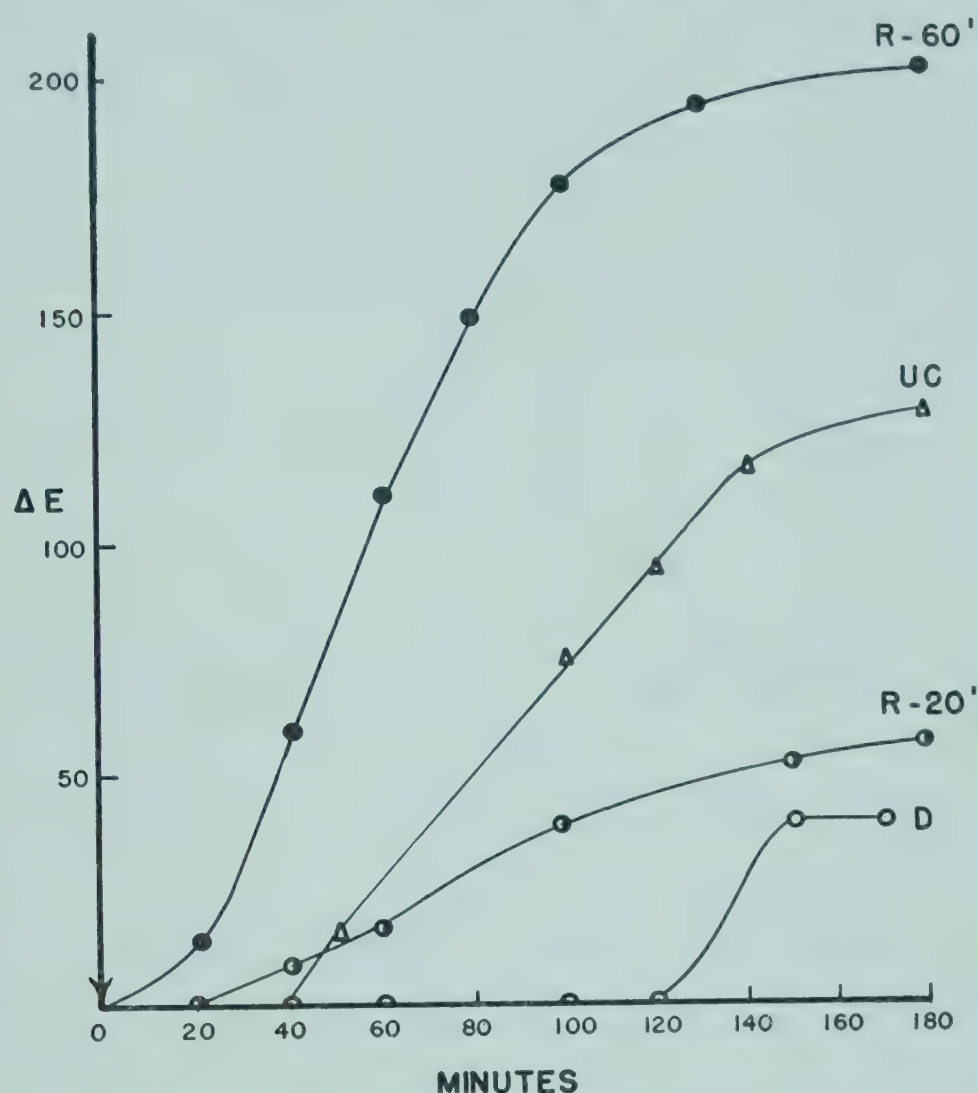


FIG. 8. The effect of depletion and replenishment of the free nucleotide pool on the synthesis of alpha-glucosidase in yeast.

Ordinates represent increments in enzyme as measured by  $Q_{CO_2}^{O_2}$  on maltose and checked by hydrolysis of (APG). Inducer (maltose) was introduced at zero time. Curve D, depleted cells; curve UC, non-depleted controls; curves R-20' and R-60', the effects on depleted cells of 20-min. and 60-min. replenishments of the nucleotide pools.

#### F. The Compulsory Coupling between Enzyme Formation and RNA Synthesis.

The data described in the preceding section suggest that the availability of a nucleotide pool, complete both as to level and composition, is a necessary prerequisite for enzyme formation.

An interesting feature of such experiments as those described in Fig. 8 emerges upon comparison, both of the rates of enzyme syn-



thesis and of the ultimate levels of enzyme content attained under the various conditions. The low *rates* of synthesis with partially replenished pools do not present a problem for explanation. It is

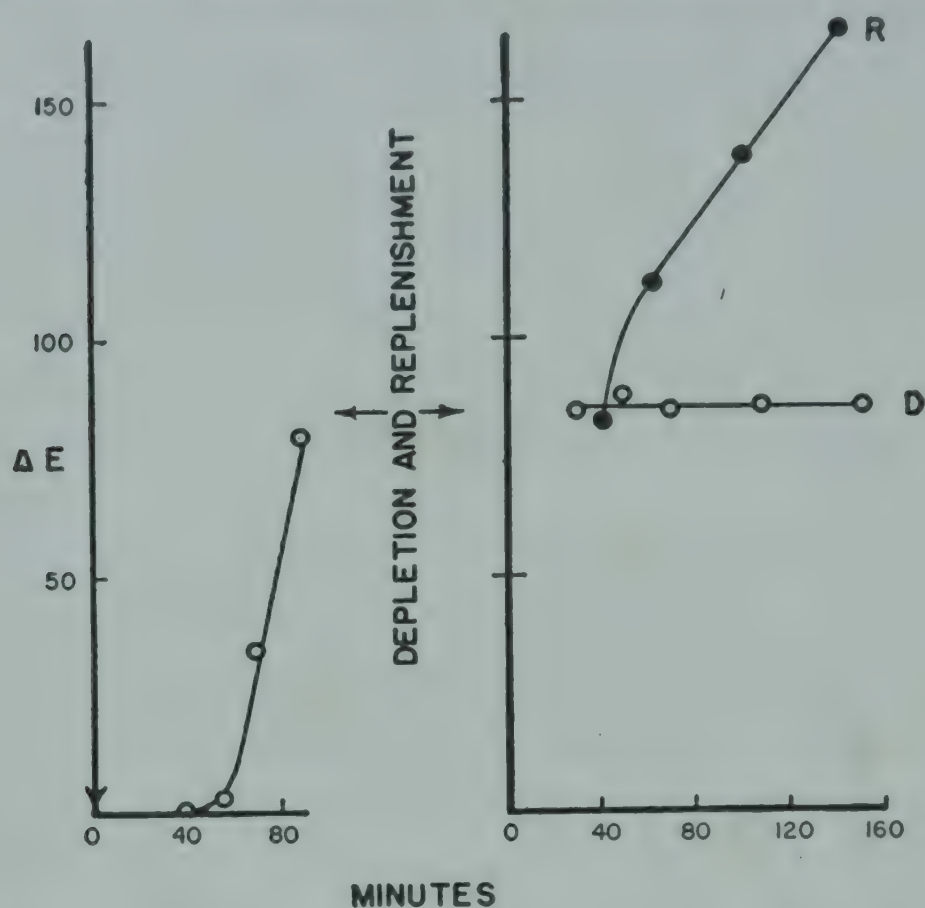


FIG. 9. The effect of removal of the nucleotide pool on alpha-glucosidase synthesis by partially induced cells.

Ordinates are the same as in Fig. 8. Cells were partially induced to the level indicated in the left-hand figure. The behavior subsequent to depletion of the nucleotide pool is described by curve D, and the effect of replenishing the depleted pool by curve R.

not, however, easy to see why such cells do not continue functioning at the lowered rates until they form enzymes in amounts comparable with those of cells containing higher pool levels. The data can, however, be explained if the formation of new enzyme molecules occurs *only* if new nucleic acid can be formed. Low levels of the nucleotide pool would then soon be exhausted and this would lead to the cessation of enzyme synthesis. We are thus led to the same conclusions from these data with yeast as were derived from the observed effects of OH-uridine on beta-galactosidase synthesis in *E. coli*.

It was of obvious interest to subject this conclusion to further test. This was accomplished by seeing whether cells with partially induced enzymes would continue to form enzyme when their pools



were depleted. The formation of alpha-glucosidase in the uracilless strain of yeast was the test system employed. Log phase cells grown in excess uracil were partially induced to adequate levels of enzyme activity and were then subjected to depletion of the nucleotide pool. Half of the resulting cells were then replenished with the usual purine-pyrimidine mixture and the other half were not. Inducer, 3 per cent maltose, was then reintroduced into both aliquots, and ability to form the enzyme was examined. The results of such an experiment are described in Fig. 9. The enzyme level attained in the first stage of partial induction is indicated by the last point in the curve on the left. It is evident that the cells which had been depleted (Curve D), although they have a fair amount of enzyme content, display no capacity to synthesize any more enzyme. On the other hand their replenishment counterparts (Curve R) immediately continue to make more enzyme on exposure to the inducer.

This experiment strongly suggests that the possession of enzyme-forming units does not, of itself, guarantee that they will function. The ability to synthesize new nucleic acid would appear to be a compulsory concomitant to the formation of new enzyme molecules.

## V. SUMMARY AND CONCLUSIONS

The behavior of the free amino acid pool of yeasts under a variety of conditions has been described. Methods have been evolved that permit the controlled variation of these pools, both as to level and composition. In the course of these studies an internal mechanism for replenishing the free amino acid pool has been uncovered. Analysis indicates that the breakdown of a labile protein containing all the amino acids is involved.

Experiments analyzing the role of the free amino acid pool in the induced synthesis of enzymes in yeast have established the following features: (a) enzyme formation is mandatorily linked to the utilization of the free amino acids; (b) the first stable intermediate on the way to an enzyme molecule is of such complexity as to demand the simultaneous utilization of all the amino acids.

The possibility that a complex preexistent precursor is convertible into active enzyme, with or without the addition of amino acids, has



been made unlikely by tracer experiments which examined the beta-galactosidase system of *E. coli*. The results obtained demonstrate that less than 1 per cent of the carbon of a newly formed enzyme molecule can come from any preexistent components, simple or complex.

The acceptance of a pathway of protein synthesis which involves the simultaneous utilization of the constituent amino acids leads rather compellingly to the consideration of a template-type mechanism. Experiments with *E. coli* and yeast are described which seek to identify the nature and mode of action of the enzyme-forming system.

The results obtained may be summarized as follows:

- (a) Drastic interference with DNA synthesis by a variety of methods is not accompanied by striking effects on enzyme formation. Such findings do not encourage postulating involvement of DNA *as such* in the enzyme-forming mechanism.
- (b) A relatively slight impairment (50 per cent) of RNA synthesis leads to complete abolishment of the enzyme-synthesizing capacity.
- (c) Purine and pyrimidine analogues can cause an immediate cessation of beta-galactosidase formation in *E. coli*.
- (d) Uracilless and adenineless mutants of *E. coli* cannot synthesize beta-galactosidase in the absence of the metabolite they require. Thymineless mutants, however, can form enzyme in the absence of added thymine.
- (e) Growth of uracilless and adenineless mutants under limiting conditions of the required metabolite leads to the temporary loss of beta-galactosidase-synthesizing ability.
- (f) The existence of a free nucleotide pool in yeast has been discovered, and methods for specific starvation of this pool devised. The effects on enzyme synthesis of varying the level of the nucleotide pool has established that its availability is a necessary prerequisite for enzyme synthesis.
- (g) Experiments with purine and pyrimidine analogues, and nucleotide pool depletion of partially induced cells have



yielded results leading to the conclusion that the synthesis of new nucleic acid is a compulsory concomitant of the continued formation of new enzyme molecules.

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# FROM AMINO ACIDS TO PROTEINS

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PROTEIN FORMS one of the major components of living matter, and the peculiar properties of living cells are largely determined by the activities and organization of the proteins they contain. Proteins are built from amino acids, of which some 24 or more have been isolated from cellular material, and the different properties of different proteins are reflections of the surfaces presented by various permutations and combinations of those amino acids arranged in convoluted polypeptide chains. Any discussion on the metabolism of amino acids must necessarily include some consideration of the means whereby those amino acids are taken up by cells, organized and orientated with regard to each other, and laid down in many different protein structures, each of specific and predetermined composition. Such consideration is fundamental to an understanding of living processes and has been the focus of much investigation, argument, and speculation. The present contribution represents an attempt to appraise what is known of the mechanisms whereby an amino acid can become part of a protein in one type of living cell: *Staphylococcus aureus*. No attempt will be made to review all the literature that has grown up around the subject as a whole, especially since a number of recent reviews are available (1, 2, 3).

In *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) we have an organism most strains of which have lost the ability to synthesize many of the naturally occurring amino acids and which are, consequently, unable to grow or synthesize protein unless these amino acids are supplied in the environment. When the organism is grown in a medium rich in amino acids, then we find that amino acids in the free state accumulate within the cells during growth. This collection of free amino acids within the cells has been referred to



as the "amino-acid pool," and the passage of amino acids into such pools together with their subsequent utilization is the subject of previous contributions to this Symposium (4, 5). Although comparatively few cells have been investigated in detail as yet, it is already clear from the work of Christensen and his colleagues (6) on mouse ascites carcinoma cells, of Spiegelman and Halvorson (7) on yeast cells, and from our own work on staphylococcal and streptococcal cells (3) that the processes concerned in controlling the concentration of a given amino acid in the pool differ widely from organism to organism. In the yeast cell, quantitative studies of the amino acids within the pool have shown that these amino acids enter into protein synthesis and are used up during adaptive enzyme formation (7), while the preformed protein of the cell apparently does not enter in any way into the formation of new protein. Spiegelman and Halvorson have produced evidence, by investigation of the utilization of the amino acid pool and inhibition of that utilization, that can be advanced in favour of the suggestion that amino acids are built directly into protein without the intermediate formation of peptides. It seems, from the work of these authors, that amino acids accumulate in the internal medium of the yeast cell as a stage in their incorporation into protein. The situation in staphylococci is not quite the same. In this organism, accumulation of free amino acids in high concentration occurs when protein synthesis is disorganized; when protein synthesis is occurring under optimal conditions there is no concomitant accumulation of free amino acids within the cell. At first sight, the situation in the two organisms might appear to be similar, amino acids being concentrated across the cell wall and then utilized for protein synthesis, the relative rates of the passage into the cell and of the incorporation into protein determining the degree of concentration in the free state within the cell. However, if the rate at which a given amino acid, say, glutamic acid, is removed from the external medium is determined in staphylococci, it is found that the rate of removal is markedly greater when the amino acid is accumulating in the free state within the cell than when it is being incorporated into new protein.



The accumulation of the free amino acid appears to be a consequence of disorganization of the protein-synthesizing mechanism. When conditions are optimal for protein synthesis, glutamic acid is removed from the external medium at the rate at which it is embodied in the new protein, and the process (free glutamic acid  $\rightarrow$  protein-glutamate) is quantitative (3). Although simple peptides can be formed when staphylococci are incubated with mixtures of two or three amino acids, there is no evidence for their formation when a complete mixture of amino acids is supplied and protein synthesis takes place smoothly (3). So in the synthesis of adaptive enzymes by yeast cells and in the formation of cellular protein by staphylococci, we observe the disappearance of amino acids and the appearance of protein composed of those amino acids. We now have to try to find out something of the path from amino acids to proteins.

A question that arises early in our consideration of this path is whether it consists of a single track or whether an amino acid can become part of a protein by more than one route. An amino acid can clearly become part of a protein by joining other amino acid residues in the course of total synthesis; is there a possibility that it can become part of a preformed protein other than by a process involving the complete breakdown and total resynthesis of that protein? Consideration of the structure of a finished protein molecule would suggest that this is improbable, but until we know the mechanism whereby the peptide bonds are formed and amino acids laid down about those bonds, we cannot be certain on this point. We might try a simple test in the following fashion: let us take a cell (such as a staphylococcus) which is unable to synthesize protein unless it is supplied with a variety of amino acids, and let us incubate it with a single amino acid labelled with  $C^{14}$ ; let us then isolate the protein of the cell and see whether the labelled amino acid has managed to get into it. This test has, of course, been carried out with many tissues under a wide variety of conditions and by several groups of workers; the answer is that the labelled amino acid is usually found in the protein of the tissue and is said to have been "incorporated" therein. Whether such incorporation involves



the same mechanism as total protein synthesis or whether there is another path from amino acids to proteins is one of the matters that must receive our attention here.

Throughout recent literature one finds many suggestions that nucleic acids play a role in protein synthesis. These suggestions arise from consideration of two main types of experimental finding: (a) that a high rate of growth, or of protein synthesis, or both, is correlated in many tissues with a high nucleic acid content and that, where it is possible to vary the nucleic acid content of cells, then the rate of protein synthesis varies with the nucleic acid content (3, 8, 9, 10); and (b) that alterations in the structure or activities of bacterial cells by transforming factors or bacteriophages are associated with transfer of desoxyribonucleic acid (DNA) to the affected cells (11, 12, 13). The possibility that protein synthesis occurs by reversal of proteolytic enzymes is not supported by any satisfactory evidence of such reversal under physiological conditions, nor is it easy to picture how the orientation of amino acids within protein chains, and the differing arrangements in specific proteins, could be explained along these lines. The nucleic acid chains can, however, be pictured as providing templates wherein specific groups of nucleotides provide combining points or niches for specific amino acids and so determine the position of these amino acids prior to their combination through peptide bonds (14, 15).

Against this background, let us consider the experimental evidence concerning the incorporation of amino acids into proteins and the synthesis of new proteins in *Staphylococcus aureus*.

#### FROM AMINO ACIDS TO PROTEINS IN INTACT STAPHYLOCOCCI

The assimilation and incorporation of amino acids by intact staphylococci have recently been reviewed elsewhere (3), and it will be necessary here only to summarize the main facts.

When staphylococci are supplied with a single amino acid and a source of energy (Condition 1), no increase in cellular protein can be measured, but the amino acid, as in the case of glutamic acid, may become concentrated to a high degree within the cell. If the



amino acid is labelled, then the label is found after incubation not only in the free amino acid within the cell but also in the corresponding amino acid residues of the cell protein. In the case of glutamic acid, both accumulation and incorporation are inhibited by substances inhibiting fermentation or uncoupling energy reactions.

When staphylococci are supplied with a source of energy and a mixture of amino acids which may be complete (i. e., contains all those found in proteins) but which must contain all those amino acids necessary for growth of the organism concerned (Condition 2), then cellular protein begins to increase. The rate of increase can be markedly accelerated by the presence of a mixture of purines and pyrimidines (PP), and under these conditions (Condition 3) the ribonucleic acid of the cells also increases. Synthesis of ribonucleic acid (RNA) occurs to a significant extent only if the cells are incubated in a medium containing all the substances necessary for optimal synthesis of protein; omission of one essential amino acid leads to cessation of synthesis not only of protein but also of RNA. No incorporation of  $C^{14}$ -thymine can be detected under these conditions (16). The rate at which protein synthesis takes place can be directly correlated with the nucleic acid content of the cells.

The rate of incorporation of a labelled amino acid into the protein fraction varies according to the conditions; under Condition 2 incorporation continues in a linear fashion for some hours, but under Condition 1 the rate of incorporation of the added amino acid is slower than under Condition 2 and decreases with time. If a mixture of amino acids, which does not contain all those essential for growth, is present, then the rate of incorporation of  $C^{14}$ -glutamic acid varies markedly with the composition of the mixture and in a manner not paralleled by changes in the cell protein; e. g., omission of aspartic acid from an otherwise complete mixture stops protein synthesis but doubles the rate of glutamic acid incorporation (17). The incorporation of labelled phenylalanine, included as one component of a complete amino acid mixture, is inhibited by *p*-chlorophenylalanine, and this inhibition is accompanied by cessation of protein synthesis although the rate of glutamic acid incorporation is unaffected (18).



Antibiotics such as chloramphenicol, aureomycin, or terramycin at limiting bactericidal concentrations effect a complete inhibition of cellular protein synthesis under Condition 2; penicillin has no effect on protein synthesis under these conditions unless relatively enormous concentrations are used. If, however, the incorporation of  $C^{14}$ -glutamic acid is studied, it is found that, although the sensitivity of the process under Condition 2 is the same to antibiotics as that of protein synthesis, under Condition 1 incorporation is relatively insensitive to chloramphenicol or aureomycin but is 50 per cent inhibited by limiting bactericidal concentrations of penicillin.

When glutamic acid is the only amino acid present, it becomes concentrated in the free state within the cells. The specific activity of the free glutamic acid within the cells decreases as the specific activity of the protein rises; and inhibition by penicillin of the incorporation prevents the decrease of specific activity of the free glutamic acid. From this evidence it would appear that the incorporation which occurs when one amino acid only is present does not take place by the same process as that involved in protein synthesis when a complete amino acid mixture is present. It seems probable that the process occurring in the former case involves an exchange between glutamic acid residues of the cellular protein and the free glutamic acid within the cell or present in the medium. Detailed investigations have been carried out only with glutamic acid up to the present, but incorporation under Condition 1 takes place with all the amino acids tested.

When this stage in the investigation was reached, it was evident that further knowledge concerning the relation between protein synthesis, incorporation reactions, and nucleic acids would await examination of these matters in a biological system less complex than intact staphylococci.

#### PREPARATION OF DISRUPTED STAPHYLOCOCCI

The incorporation of labelled amino acids can be detected with considerable sensitivity and can be estimated accurately. Attempts were therefore carried out to prepare a non-viable fraction of staphy-



lococci which could incorporate  $C^{14}$ -glutamic acid. After numerous methods of cell disintegration had been tested, active material was eventually obtained by exposing thick suspensions of washed staphylococci in buffered sucrose solution to supersonic vibration at 25 kilocycles/sec., and fractionating the disintegrated material on the centrifuge. The period of exposure to supersound varies from 15–40 min., according to the efficiency of the transducer assembly in use and according to the degree of resolution (with regard to nucleic acid, see below) required. The disintegrated preparation is first centrifuged for 7–8 min. at 800 g to remove any intact or slightly damaged cells. The supernatant is turbid, and the greater part of the material responsible for this turbidity can be centrifuged down at 4000 g in about 20 min. The sediment so obtained, after washing and suspension in phosphate buffer, is able to incorporate  $C^{14}$ -glutamic acid if incubation takes place in the presence of a source of energy such as adenosinetriphosphate (ATP) and hexosediphosphate (HDP) (19). The finer particles and the soluble portion of the disintegrate are devoid of activity; the soluble portion has been used for preparation of staphylococcal nucleic acid. Examination in the electron microscope of the active sediment shows that it consists of broken cell envelopes with a variable amount of electron-dense material remaining in their centres; the amount of this material decreases with the time of exposure to supersonic vibration. The "cells" are found to have lost 50 to 70 per cent of their protein and nucleic acid; they are non-viable and have no respiration but are able to form acid from glucose and to utilize ATP and HDP as energy sources for incorporation reactions or protein synthesis.

Nucleic acids can be extracted from these broken cells by such treatments as incubation with  $M$  NaCl, ribonuclease (RNAase) or desoxyribonuclease (DNAase). Such treatments result in further removal of both nucleic acid and protein from the "cells" and, on occasion, it has been possible to remove almost all the nucleic acid from the "cells" by one or the other of these treatments. Electron microscope examination of preparations then shows that only small amounts of electron-dense material remain, either spread diffusely over the cell envelope or sometimes collected in a small mass in the centre.



INCORPORATION OF  $C^{14}$ -LABELLED GLUTAMIC ACID  
BY DISRUPTED CELLS

Incubation of the disrupted cell fraction with  $C^{14}$ -glutamic acid and a source of energy such as glucose or ATP + HDP results in incorporation of the radioactive amino acid into the protein of the fraction (19). In order to avoid possible confusion of results due to contamination of the fraction with intact cells, it has been our practice to use ATP + HDP as energy source in all this work since intact cells are unable to utilize this mixture. As with intact cells, the course of the incorporation is markedly influenced by the presence of a complete mixture of amino acids (see Fig. 1). When this is present, incorporation proceeds in a linear fashion for some hours; when glutamic acid is the only amino acid present (Condition 1), incorporation ceases after a time and at a level which depends upon the concentration of glutamic acid in the medium. Incorporation under Condition 2 is accompanied by a significant increase in protein-nitrogen; that which occurs under Condition 1 either by no change or by a small decrease in protein-nitrogen.

The experiments carried out with intact cells suggested that the incorporation which occurs under Condition 1 might arise as the result of an exchange reaction. If this were so, it should be possible to remove radioactive glutamic acid from the protein (after previous incorporation) by incubation with unlabelled glutamic acid. Accordingly, disrupted cells were incubated with ATP, HDP, and 0.1  $\mu$ mole  $C^{14}$ -glutamic acid/ml. until the radioactivity of the preparation reached a steady value. The preparation was then washed free from radioactive glutamic acid, divided into a number of equal fractions, and each of these incubated with some combination of ATP, HDP,  $C^{12}$ -glutamic acid,  $C^{14}$ -glutamic acid or  $C^{12}$ -aspartic acid. The fraction incubated with ATP + HDP alone showed no change in radioactivity; that incubated with ATP, HDP, and  $C^{14}$ -glutamic acid showed a small increase in radioactivity; that incubated with ATP, HDP, and  $C^{12}$ -glutamic acid lost 40 per cent of its radioactivity in 90 min. Fractions incubated with  $C^{12}$ -glutamic acid alone or with

$C^{12}$ -aspartic acid and ATP + HDP showed no change in radioactivity (19). It is clear that exchange reactions can occur between protein-glutamic acid residues and free glutamic acid in the presence

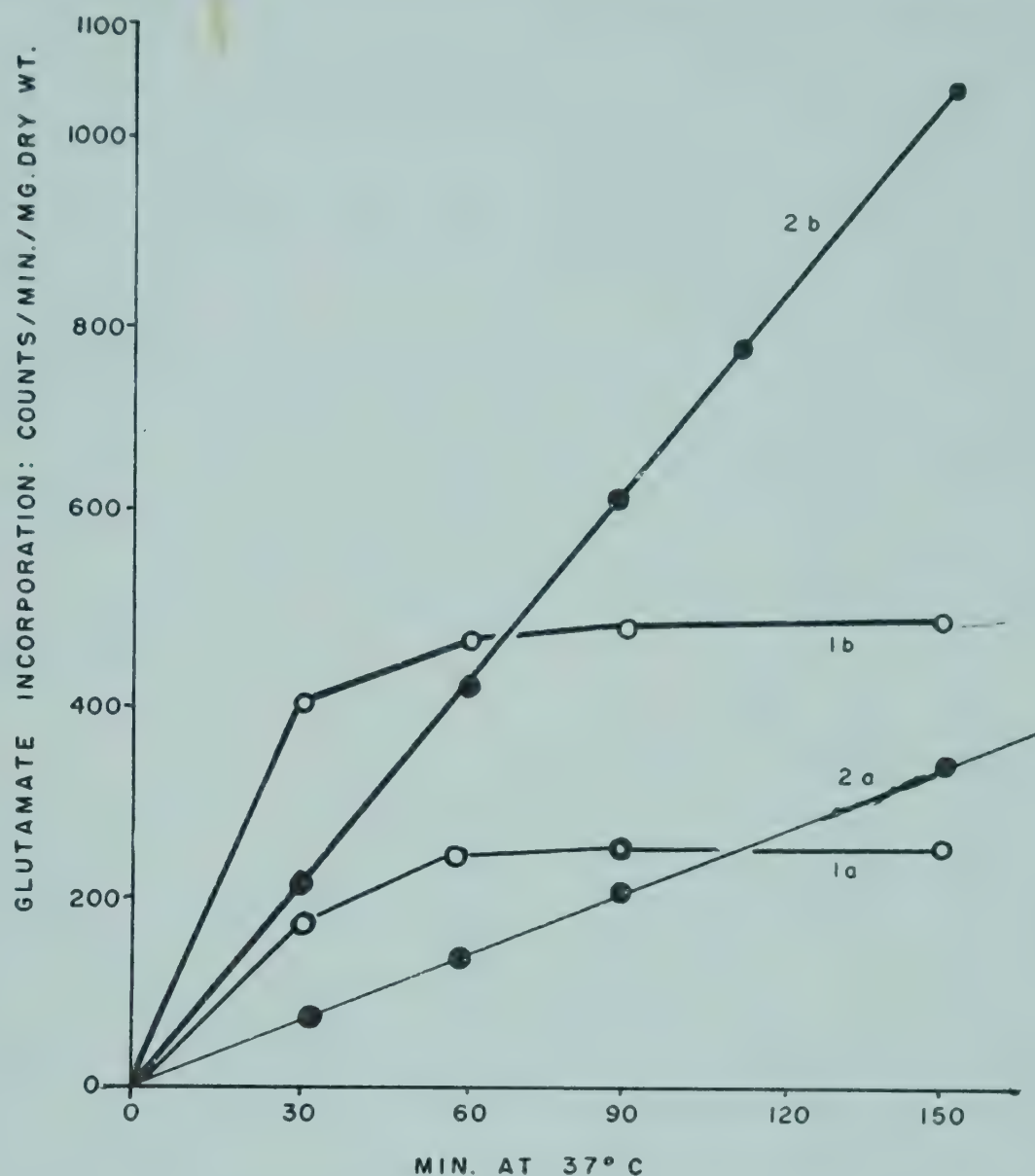


FIG. 1. Course of incorporation of  $C^{14}$ -glutamic acid into disrupted cell preparations.

Curves 1a and 1b: Salt-resolved preparation incubated with buffered saline, ATP, HDP, and  $C^{14}$ -glutamic acid.

1a, without added nucleic acid; 1b, with 0.1 mg. nucleic acid added per ml.

Curves 2a and 2b: As for curves 1, with further addition of complete mixture of amino-acids.

2a, without added nucleic acid; 2b, with 0.1 mg. nucleic acid added per ml.

Activities estimated on TCA-precipitated fraction of preparations.

of ATP and HDP, and that incorporation under Condition 1 may arise as a result of such exchanges. It is possible that some of the protein of the preparation may break down in the course of the experiment and be resynthesized with the consequent embodiment



of glutamic acid supplied in the medium. Since, however, there is insignificant loss of radioactivity from the fraction incubated with ATP + HDP alone or with  $C^{12}$ -glutamic acid alone there can be little breakdown of the protein involved in the exchange reaction

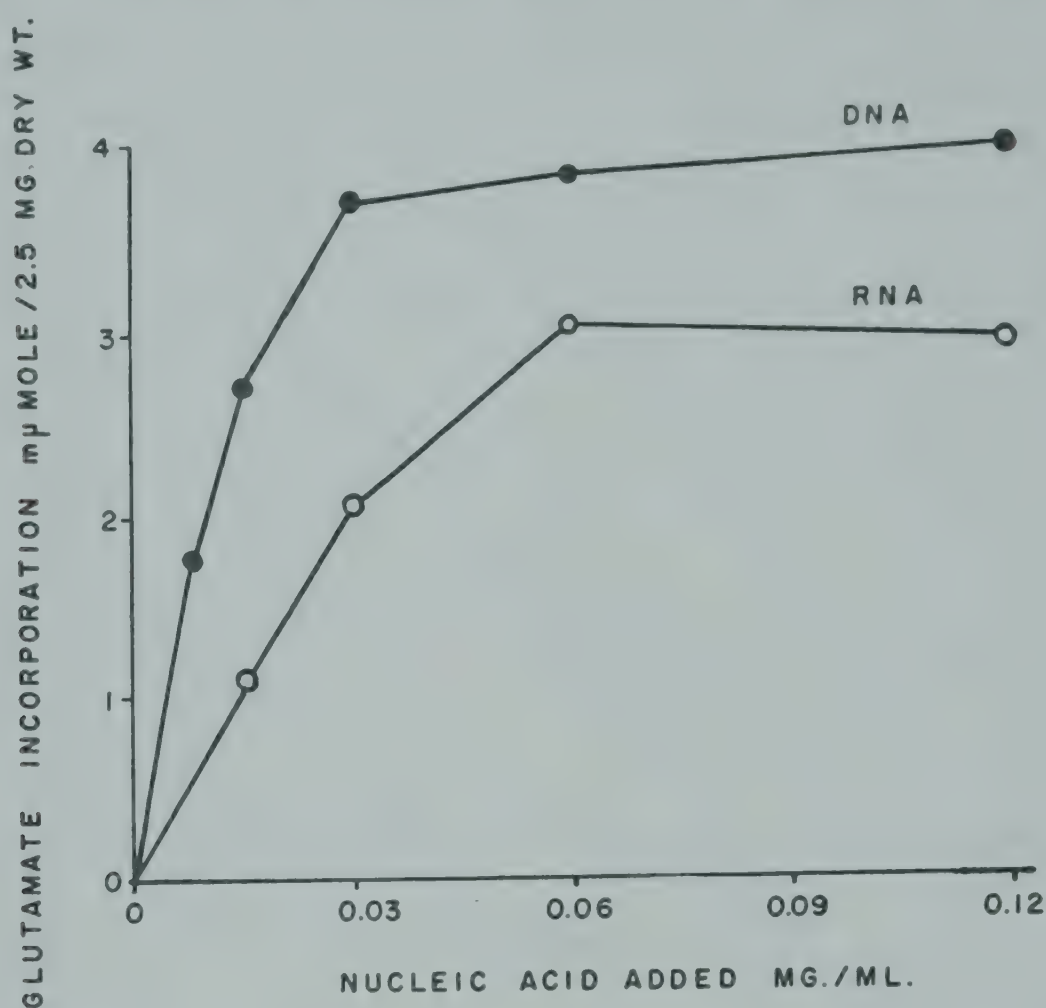


FIG. 2. Effect of nucleic acids on rate of incorporation of  $C^{14}$ -glutamic acid by salt-resolved disrupted cell preparation.

during the course of the experiment under the conditions used. Podolsky (20) has recently observed that the rate of protein degradation in cultures of *Escherichia coli* under a variety of conditions amounted to  $8 \pm 2\%$  in 30 hr. It seems probable that exchange reactions are limited to certain proteins within the cell; under optimal conditions incorporation of  $C^{14}$ -glutamic acid ceases when the amount of glutamic acid taken up corresponds to 5-6% of the total protein-glutamate residues in the preparation, and, as will be shown below, exchange reactions only occur in the presence of nucleic acid, so that it may be the nucleoproteins only which are involved.

Whether the incorporation of glutamic acid takes place under Condition 1 or 2, it is markedly affected by removal of nucleic acid

from the disrupted cell preparation (see Fig. 1). The rate of incorporation and the amount of glutamic acid incorporated at equilibrium can be restored by addition of either DNA or RNA prepared from the soluble portion of the staphylococcal disintegrate, although DNA prepared from thymus, herring roe, or wheat germ is inactive, as is RNA prepared from beef liver. Fig. 2 shows the effect of concentration on the activation of glutamic acid incorporation, and it can be seen that, on a dry weight basis, DNA is approximately twice as effective as RNA. The relative efficiencies of the two nucleic acid fractions are not affected by the method used to resolve (i. e., remove nucleic acid from) the disrupted cell preparation: the ratio of DNA to RNA activation is the same whether resolution has been accomplished with  $M$  NaCl, RNAase, or DNAase treatment. A mixture of purines and pyrimidines has no significant activating effect under these conditions.

*Incorporation of other amino acids; effect of time of exposure to supersound on degree of resolution.* Incorporation of amino acids under Condition 1 is not restricted to glutamic acid but takes place with all natural amino acids so far tested. When, however, the effect of resolution and reactivation with nucleic acid is investigated with different amino acids, it is found that the effect of addition of nucleic acid varies with the amino acid tested and with the time for which the disrupted cells were exposed to supersound during preparation. For example, if we take salt-resolved disrupted cells and incubate them with a single amino acid with and without a saturating concentration of nucleic acid, we find, in a specific instance, that the activation of incorporation by nucleic acid is: glycine 550%, aspartic acid 210%, glutamic acid 150%, lysine 20%, and alanine 0%. However the cells are treated for the purpose of resolution, the response to nucleic acid varies from amino acid to amino acid, and, up to the present, no procedure has resulted in any effect of nucleic acid being demonstrable on the incorporation of alanine. The degree of resolution in any one case increases with the time of exposure to supersound during preparation. Fig. 3 shows results obtained for the incorporation of a number of amino acids by preparations made



by exposing cells for periods varying from 15–55 min. in a transducer assembly. With more efficient assemblies, the time of exposure required to give a particular resolution may be reduced considerably

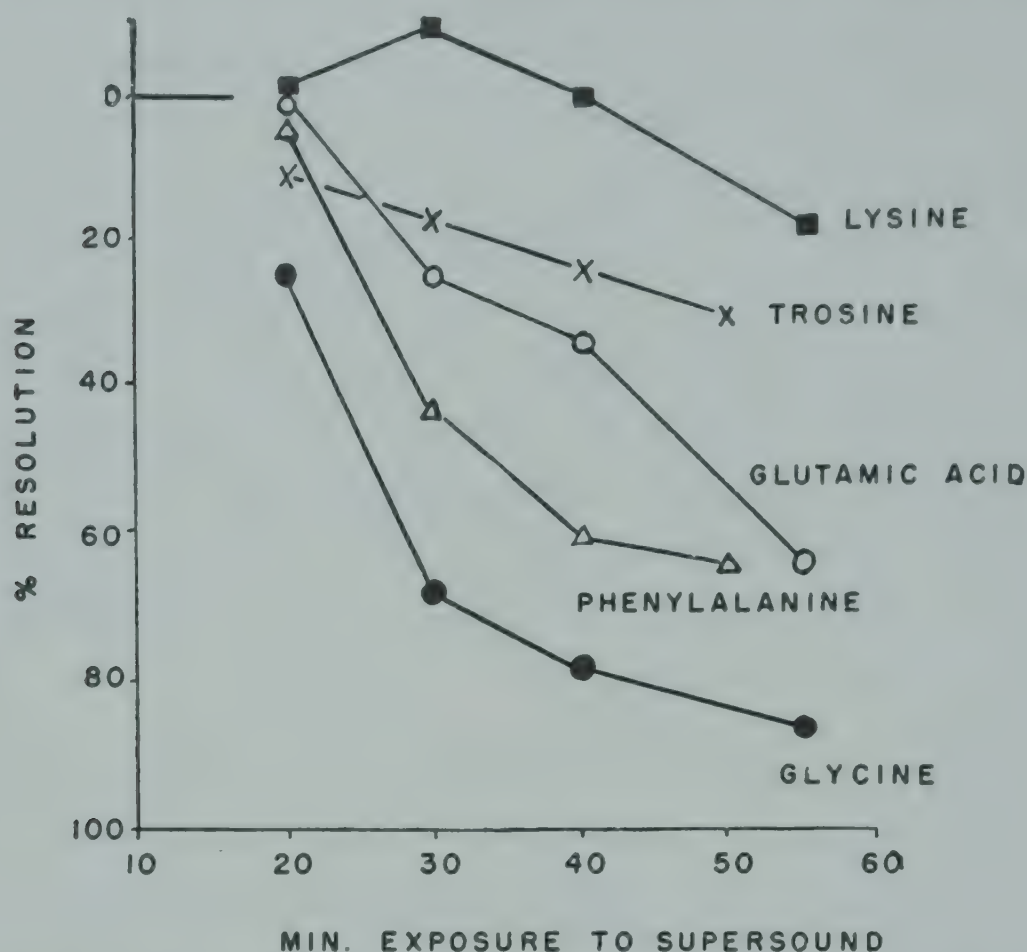


FIG. 3. Effect of duration of exposure to supersonic vibration on subsequent resolution of disrupted cell preparation with respect to incorporation of various amino acids.

If rate of incorporation in presence of saturating concentration of nucleic acid =  $A$  and rate in absence of added nucleic acid =  $B$  then % resolution =  $100 \frac{(A-B)}{A}$ .

These results have been obtained by testing incorporation under Condition 1, and it is clearly of interest to know whether similar variations between amino acids occur under Condition 2. Fig. 1 shows that nucleic acid has a marked effect on the rate of incorporation of  $C^{14}$ -glutamic acid under Condition 2; an experiment has been carried out under this condition by using a complete mixture of amino acids in which different amino acids have been labelled in parallel but otherwise identical samples. It was again found that the effect of nucleic acid varied with the amino acid whose incorporation was measured, glycine again showing the largest stimulation, while alanine was unaffected by the presence of nucleic acid. If

incorporation under Condition 2 was a measure of protein synthesis, it would be expected that a change in the rate of such synthesis would be accompanied by similar changes in the rates of incorporation, whatever amino acid were being studied. Either the method is not suitable for the study of protein synthesis as such, or the effects of nucleic acids on protein synthesis are related to specific amino acids rather than to specific proteins. We have already had many indications that the former is true; consequently we have abandoned the use of isotopes and incorporation studies as means of studying protein synthesis *per se*, and have turned our attention instead to changes in protein-nitrogen and in specific enzymes in the disrupted cell preparation (21).

#### EFFECT OF NUCLEIC ACIDS ON ENZYME SYNTHESIS IN DISRUPTED CELLS

The amount of disrupted cell preparation available for investigation after the normal disintegration and resolution procedures is small, and it follows that it is only possible to test the development of enzymes which either have high activity or have activity which can be measured with considerable sensitivity. So far we have investigated the development of catalase, the system producing acid from glucose (which for brevity we will call "glucozymase"), and  $\beta$ -galactosidase.<sup>1</sup> In general, salt-resolved disrupted cells have been incubated with ATP, HDP, a complete mixture of amino acids, and RNA, DNA, or a mixture of purines and pyrimidines (PP); centrifuged down after 90-120 min. at 37° C.; resuspended in a suitable buffer, and their enzymic activities determined (21). In each of the cases studied, it has been possible to demonstrate a significant development of activity in the disrupted cells and, also in each case, to show an effect of nucleic acids upon that development, as summarized in Fig. 4. Changes in protein-nitrogen of the preparation are not as marked as some of the increases obtained with enzymes, probably because the preparation contains non-functional

<sup>1</sup> Pronounced bē-tə gə-lăc-tō-sī-dăse.



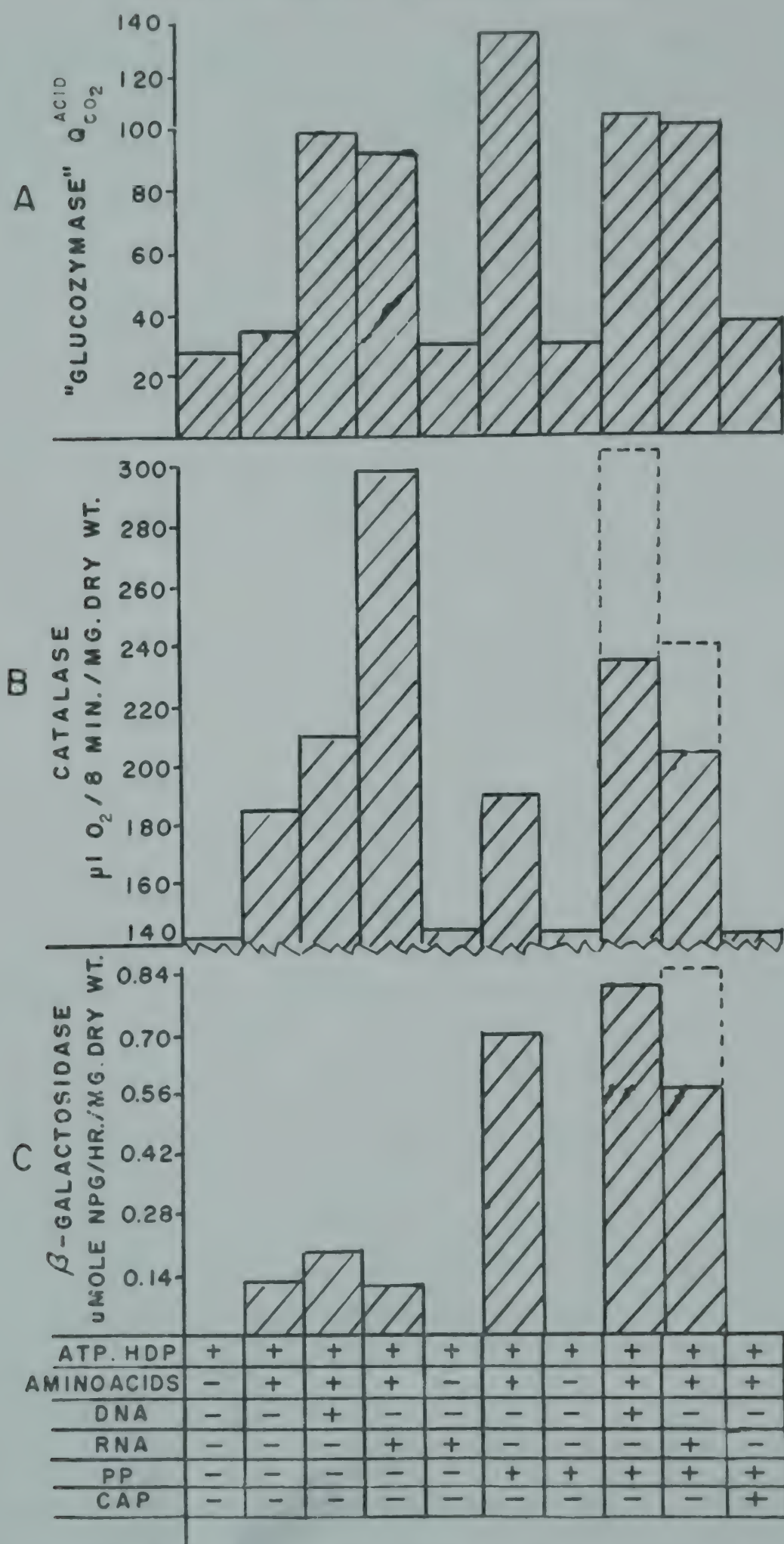


FIG. 4. Conditions affecting the development of certain enzymic activities in salt-resolved disrupted cell preparations.

A, Glucozymase development; incubation 90 min. at 37° C.

B, Catalase development; incubation 90 min. at 37° C.

C,  $\beta$ -Galactosidase development; incubation 120 min. at 37° C.; 2% galactose present in all incubation mixtures.

Concentrations 1 ml.: ATP, 2  $\mu$ mole; HDP, 20  $\mu$ mole; amino acid mixture 0.5 mg. each component; DNA, 0.02-0.1 mg.; RNA, 0.03-0.1 mg., PP, 25  $\mu$ g. each component; chloramphenicol (CAP), 30  $\mu$ g.

"protein"-nitrogen in the cell wall. Incubation of one preparation for 90 min. with amino acid mixture and ATP + HDP alone, or in the presence of DNA, RNA, or PP gave rise respectively to 2, 21, 31, and 20% increase in protein-nitrogen.

The investigations on amino acid incorporation have shown that the degree of resolution of the disrupted cell preparation increases with the time of exposure to supersound. The same effect has been obtained with the effect of nucleic acids on protein synthesis. When the time of exposure is short, protein synthesis occurs readily if the preparation is incubated with ATP, HDP, and amino acids, and the addition of nucleic acids or PP has little effect. As the exposure is increased, protein synthesis in the absence of added nucleic acid decreases but can be restored by the addition of the appropriate nucleic acid or PP (as discussed below). When exposure is prolonged to a stage where the yield of disrupted cells is less than 1% of the initial suspension, resolution is high, electron-microscope examination shows little dense material in the broken envelopes, and protein synthesis in the absence of added nucleic acid is abolished or insignificant but can be restored by adding nucleic acid. In general, it is possible to show clear-cut effects of RNA or PP at the intermediate stage of partial resolution, while DNA effects become apparent at the final stage of high resolution.

*Glucozymase.* Fig. 4 shows the development of "glucozymase" activity in disrupted cells at the stage of high resolution. The initial activity of the preparation is small, and little increase in this activity occurs if the preparation is incubated for 90 min. with ATP, HDP, and amino acids. The addition of PP, DNA, or RNA greatly stimulates the development of activity, PP being the most effective of the three additions. Omission of the amino acid mixture or addition of 30  $\mu$ g. chloramphenicol/ml. (which inhibits protein synthesis) abolishes the development of activity in all cases; consequently it is reasonable to assume that the development is due to synthesis of protein. Addition of either DNA or RNA together with PP gives stimulation of development less than that obtained with PP alone. The concentrations of DNA and RNA required to



give optimal stimulation are of the same order as those required to saturate glutamic acid incorporation (see Fig. 2). The glucozymase system is a complex one involving the development or activation of many enzymes, and clearer results have been obtained with studies of single enzymes, such as catalase or  $\beta$ -galactosidase.

*Catalase.* The disrupted cell preparation contains a high catalase activity initially, but it has been possible to show a doubling of this activity over 90 min. incubation under suitable conditions. Fig. 4 shows the development of catalase activity in preparations at the stage of partial resolution. Incubation with ATP, HDP, and amino acid mixture results in some increase, and this is markedly stimulated by the addition of RNA to the medium. In this case, PP has little or no stimulating effect; on occasions it has depressed the activity below that obtained with amino acid mixture alone. The addition of RNA and PP together is less effective than RNA alone, whereas DNA + PP is more effective than either alone; on two occasions DNA + PP proved to give a stimulation greater than that given by RNA. The effect of DNA depends upon the degree of resolution of the preparation; at the stage of partial resolution shown in Fig. 4, DNA has little effect but, as resolution increases, the effect of RNA decreases whereas DNA becomes relatively more effective. At the stage shown in Fig. 4, the addition of RNA and DNA together is no more effective than RNA alone; at high resolution, the effects of RNA and DNA become additive. The concentration of DNA appears to be critical; in a specific instance 0.6 mg. of highly resolved disrupted cells as activated by 0.02–0.035 mg. DNA/ml., whereas 0.08 mg. DNA/ml. was without significant effect. The stimulation produced by either DNA or RNA is abolished by the addition of RNAase during incubation.

*$\beta$ -Galactosidase.*  $\beta$ -Galactosidase is an adaptive enzyme whose properties and formation in *E. coli* have been the subject of a series of brilliant studies by Monod and Cohn (22). It is also adaptive in *Staph. aureus*, although many of the properties of the enzyme in this organism differ from those described for the enzyme of similar



function in *E. coli* (23). Enzyme activity has been estimated by the colorimetric method based upon its action on nitrophenylgalactoside (24). The initial activity of preparations of disrupted cells originally grown in the absence of either lactose or galactose was zero, and no development of  $\beta$ -galactosidase took place unless an inducer (22) was added to the incubation mixture. If the preparation is incubated with galactose as inducer, amino acid mixture, and PP, there is a development of  $\beta$ -galactosidase activity which continues, after a short lag period, in a linear fashion for up to 3 hr. In partially resolved preparations, the amino acid mixture in the absence of PP is found to give rise to enzyme formation at a rate 30–45 per cent of that obtained in the presence of PP and, at this stage, addition of neither DNA nor RNA has any significant effect. In no case has RNA shown any stimulating effect when added alone, although, in a small proportion of tests, a high concentration of RNA prepared from cells grown in galactose has increased the effect of PP. As the time of exposure to supersound is increased, the response of the resolved preparation to amino acids decreases (see Fig. 5), while the total activity evoked by amino acids and PP also decreases. As the resolution increases, an effect of DNA becomes apparent; Fig. 4 shows a result typical of partially resolved preparations where DNA has a small stimulating effect over that obtained with amino acids alone, and also increases the effect due to PP. Fig. 5 shows that when the response to amino acids alone has become small, a very significant stimulation by DNA can be demonstrated; DNA prepared from galactose-grown cells is 2 to 3 times more effective on a dry weight basis than DNA from glucose-grown cells. In the most highly resolved preparations made (corresponding to time 40 min. in Fig. 5) "adapted" DNA from galactose-grown cells has a very marked effect, whereas neither amino acids nor PP alone, nor both together, have any significant effect; DNA + PP is no more effective than DNA alone at this stage, but with preparations removed from the transducer at a stage slightly before this (time 35 min. in Fig. 5) DNA + PP is more effective than either alone. Treatment of such preparations with DNAase abolishes the



response to PP, but this response can be fully restored by addition of 0.015 mg. adapted DNA/ml. If RNase is added to the incubation mixture during development of  $\beta$ -galactosidase in highly resolved preparations, the response to both DNA and PP is abolished or greatly decreased.

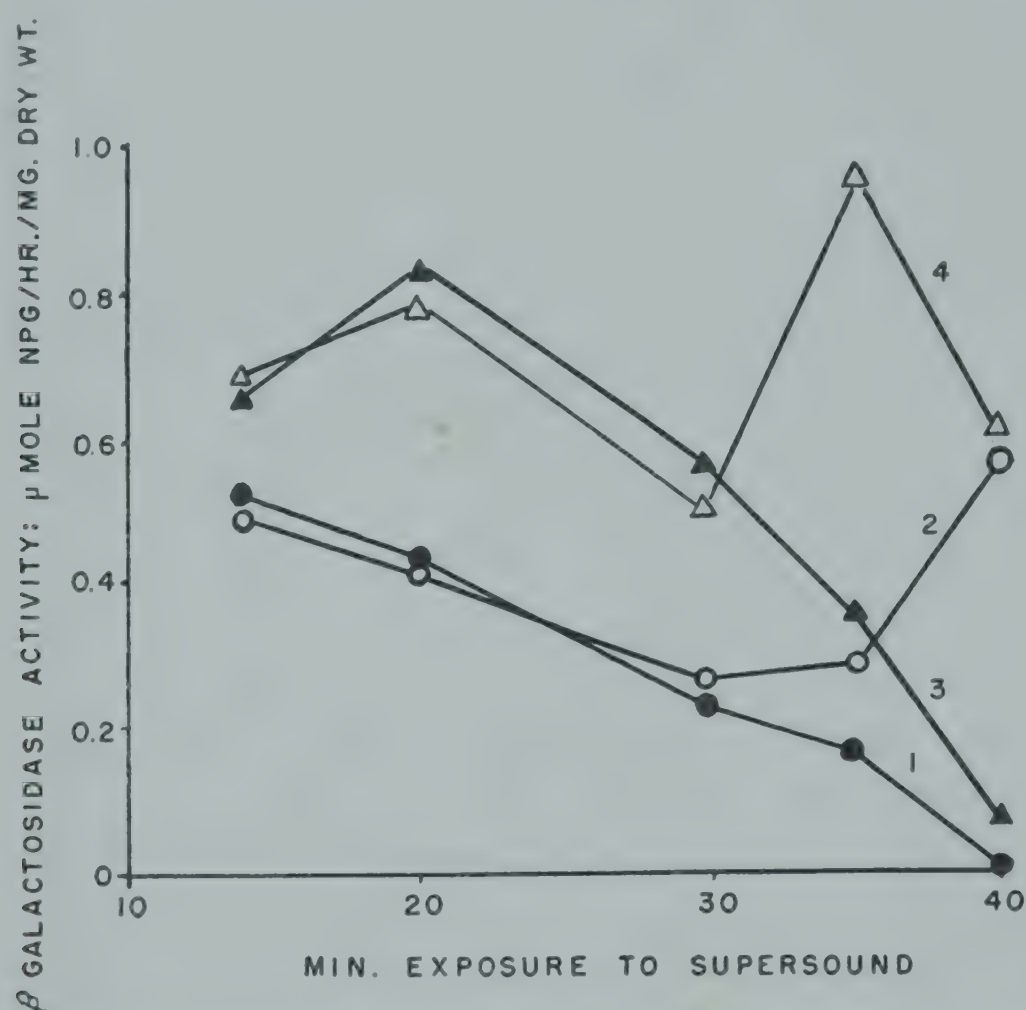


FIG. 5. Effect of duration of exposure to supersonic vibration on development of  $\beta$ -galactosidase in salt-resolved disrupted cell preparations incubated with ATP, HDP, and the following: Curve 1, amino acid mixture. Curve 2, amino acid mixture + 0.02 mg. DNA (from galactose-grown cells)/ml. Curve 3, as curve 1 + PP. Curve 4, as curve 2 + PP.

*RNA synthesis.* The effect of PP on the development of  $\beta$ -galactosidase and glucozymase would suggest that the disrupted cells are able to synthesize nucleic acids under the conditions used in these experiments, just as intact cells are able to synthesize ribonucleic acid when supplied with a source of energy, a complete mixture of amino acids, and PP. This has been tested by including  $C^{14}$ -labelled uracil or thymine in the PP mixture and determining their incorporation into the nucleic acid fraction of the disrupted cells after incubation under the various conditions used in these experiments. No

incorporation of thymine has been obtained under any of the conditions tested. Incubation of disrupted cells with  $C^{14}$ -uracil, ATP, HDP, and either amino acids or PP separately resulted in a small incorporation corresponding to less than 1 m $\mu$ mole uracil/mg. dry weight of preparation/hr.; the presence of amino acids and PP together gave incorporation of 3-4 m $\mu$ mole uracil/mg./hr., while addition of galactose to the medium, as for the development of  $\beta$ -galactosidase, resulted in 5 to 6 times increase in the uracil incorporation. When the course of uracil incorporation is followed during  $\beta$ -galactosidase development, it is found that incorporation is most rapid during the first 30 min. of the incubation period, and this period of rapid ribonucleic acid synthesis precedes the onset of  $\beta$ -galactosidase formation. The formation of  $\beta$ -galactosidase in *Staph. aureus* is inhibited by penicillin in low concentrations (21, 23); a concentration of 30 units penicillin/ml. inhibits  $\beta$ -galactosidase formation in disrupted cells by 80 per cent and the corresponding uracil incorporation by 65 per cent.

#### AMINO ACIDS—NUCLEIC ACIDS—PROTEINS

It is immediately clear that nucleic acids are concerned in protein synthesis, and experimental proof is now available of the often postulated interrelationship. It is too early to deduce definite roles for the two types of nucleic acid in general protein synthesis, as the experimental system is still crude and the number of syntheses which can be studied so far is small and may represent special cases. Nevertheless much of the fun in science and most of the arguments in symposia arise from speculation and hypothesis building, and some such exercise would not be out of place here. The impression gained from studies on catalase and  $\beta$ -galactosidase formation is that RNA plays a key role in the final elaboration of specific proteins. This is clear in the case of catalase, and appears to underlie the effects obtained with  $\beta$ -galactosidase where, in partially resolved preparations, PP markedly stimulates enzyme formation, the stimulation being accompanied by RNA synthesis and abolished by RNAase.  $\beta$ -Galactosidase formation cannot be stimulated by RNA



preparations even when these are made from galactose-grown cells, and it may be that enzyme formation in this case is dependent upon a RNA which is unstable except in the presence of an inducer. Since PP is not effective in stimulating catalase formation it would appear that the disrupted cells are not able to synthesize this particular RNA from PP with any readiness. A further difference between the catalase and  $\beta$ -galactosidase systems is that disrupted cells have a high catalase activity initially, so that the protein model must be relatively abundant in this case from the beginning.

Experiments with highly resolved cells show that the presence of RNA is certainly not the whole story in the control of protein synthesis since, in such preparations, DNA is frequently more effective than RNA or PP. The effect of DNA in both catalase and  $\beta$ -galactosidase development is abolished if RNAase is present during incubation; this, taken together with the fact that DNA markedly affects the response to PP in  $\beta$ -galactosidase development, suggests that DNA organizes RNA synthesis and, as such, provides the initial model on which, first RNA, and then protein, synthesis takes place. A question which is not answered is whether the initial model is a DNA molecule or a DNA-protein complex. The experiments on amino acid incorporation may be relevant in this connection. It appears that some combination occurs between certain proteins and nucleic acids which results in the individual amino acid residues in those proteins becoming exchangeable with corresponding amino acids in the medium. Both RNA and DNA can bring about this condition, and it is probable that both RNA and DNA can combine with certain proteins. It is only possible to speculate about the mechanism of this exchange reaction at the present time, but it seems that the various suggestions that have been advanced concerning the actions of nucleic acids as templates for protein synthesis (3, 14, 15) can provide a feasible explanation. According to such hypotheses, specific combinations of nucleotides within the nucleic acid chain provide combining points for specific amino acids. When all the positions along the chain are filled, a peeling-off reaction takes place with the formation of



peptide bonds between adjacent amino acid residues and liberation of the polypeptide from the nucleic acid. Two phases are involved in such a reaction: first, combination of amino acids (possibly in an activated form) with nucleotide residues; then, combination of amino acid residues with each other through peptide bonds. In the first phase, amino-acid residues on the nucleic acid chain could be expected to exchange with corresponding residues in the medium. If the system were reversible so that preformed protein could combine with nucleic acid and its residues enter the first phase, then incorporation of radioactive amino acids added to the medium could occur by exchange. Such incorporation would be restricted to proteins which could undergo reversible combination with nucleic acid in this sense. The varying degree of resolution obtained for incorporation of different amino acids is difficult to explain on this picture, unless the structure responsible for rendering a given amino acid exchangeable is smaller than the complete nucleic acid and some of the structures are more readily damaged or removed than others.

A number of hypothetical schemes can be built around this framework and, in fact, many such schemes have been bandied about between the initiated during recent years. One of these schemes, with which none of the present experimental evidence is at variance, can be formulated in the following way: DNA combines with amino acids and determines their positions relative to each other prior to the formation of a specific polypeptide structure; the residues cannot combine with each other while in contact with DNA but must first be taken over by a corresponding RNA structure; this may be accomplished by combination with a preexisting specific RNA or by synthesis of RNA, from nucleotide residues, modelled on the DNA-protein complex; once RNA is available, the amino-acid residues can then combine with each other through peptide bonds in positions determined by the initial DNA template, a peeling-off reaction liberates the polypeptide structure, and protein synthesis takes place. This hypothesis would explain why RNA synthesis will occur only when a full complement of amino acids is present, and also why penicillin, which prevents the exchange



of specific amino-acid residues (3), should give rise to inhibition of both specific protein synthesis and specific RNA synthesis.

Whatever may be the final elucidation of this matter, it can now be stated as an experimental fact that the path from amino acids to proteins, whether by exchange or synthesis, involves a confluence of those amino acids and those proteins and the nucleic acids of the cell.

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# ASPECTS OF ENZYME FORMATION

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THIS SESSION is concerned with the problem of the conversion of amino acids to proteins. Dr. Spiegelman has discussed his elegant studies on the role of the free amino-acid pool in enzyme formation and Dr. Gale has discussed his very interesting work dealing with the characteristics of enzyme formation in fractured cells. I would like briefly to mention two other characteristics of enzyme formation which I feel are pertinent to the present discussion. Most of this work was carried out by Dr. Rickenberg of my laboratory (3). While the gene is an obvious and here neglected component of the protein-forming system, I will in deference to the present group limit my remarks to non-genetic aspects.

In recent years there has been a good deal of speculation about the possibility that overall cell growth is a necessary prerequisite for enzyme formation. This view, in fact, appeared to be strengthened by the recent work of Monod and his collaborators (1) on the formation of  $\beta$ -galactosidase<sup>1</sup> in *Escherichia coli*. Monod et al. found that if  $\beta$ -galactosidase is formed under conditions such that the presence of the enzyme is not essential for growth, the  $\beta$ -galactosidase then forms a constant proportion of all the cellular protein formed. Consideration of their extensive data could well suggest that  $\beta$ -galactosidase is formed only under conditions that permit the formation of all cell constituents. If one examines the formation of this enzyme in *E. coli* under conditions other than those of "gratuité," however, one finds that a quite different relationship may obtain between  $\beta$ -galactosidase formation and general cellular protein formation. Under conditions of diauxie (glucose-lactose or glucose-melobiose),  $\beta$ -galactosidase is formed during the diauxic

<sup>1</sup>Pronounced bā-tə gə-lăc-(to)-sĭd-ăse.



growth lag, yet no detectable increase in total protein can be observed. A detailed examination of  $\beta$ -galactosidase formation during diauxie suggests that during the diauxic lag synthesis of protein in general comes to an almost complete halt, while  $\beta$ -galactosidase is synthesized at a constant and maximum rate. Thus, under certain conditions, it can be shown that  $\beta$ -galactosidase may be formed in the absence of detectable growth, and preferentially with respect to other proteins.

Integrated cell growth therefore does not appear to be a necessary prerequisite for enzyme synthesis. If we assume that  $\beta$ -galactosidase is formed under these conditions in a manner similar to that discussed by Spiegelman, the fact that preferential synthesis can occur suggests that the conversion of free amino acids to a specific enzyme may represent a separate and specific process for each enzyme formed.

The second characteristic of enzyme formation which I wish to mention deals with the characteristics of the enzyme in the intact cell, and my remarks again will deal with the  $\beta$ -galactosidase of *E. coli*. It was first noted by Lederberg (2) that a sizable discrepancy is found between the  $\beta$ -galactosidase activity found in extracts of fully adapted cells and that of the intact cells from which the extracts were prepared. This discrepancy, Lederberg found, could be accounted for in part by the fact that the substrate concentration required to give a half maximal rate of hydrolysis was five times greater for intact cell  $\beta$ -galactosidase than for extract  $\beta$ -galactosidase. We have confirmed this observation and have further found that the barrier giving rise to the difference in substrate concentration required to give a half maximal rate of hydrolysis is abolished only under conditions that lead to disruption of the cells. This barrier is further unaffected by temperature or by penicillin, and is independent of the cellular  $\beta$ -galactosidase content. From what we know of this barrier at the present time it seems reasonable to conclude that it is concerned with the ability of the substrate to penetrate to the enzyme site. The effectiveness of this type of barrier can apparently be affected by gene mutations, inasmuch as a mutant unable to grow on lactose has been found to have a greatly exaggerated barrier of



this general type. The increase in the effectiveness of this barrier appears to account for the characteristics of the mutant strain, rather than any alteration in its characteristics of enzyme induction or formation.

A second barrier masking the full expression of the  $\beta$ -galactosidase activity of intact cells can also be shown. If fully adapted cells are examined, and the first barrier I mentioned is compensated for by using five times the amount of substrate in estimating intact cell  $\beta$ -galactosidase activity as is used for estimating extract activity, one still finds a substantial discrepancy between whole-cell and cell-extract activity. Under these conditions extracts show about fifteen times more  $\beta$ -galactosidase activity than is found in the cells from which the extracts are prepared. This second barrier has been termed the "Factor" and is defined operationally in terms of a ratio of extract and intact-cell  $\beta$ -galactosidase activity at saturating concentrations of the substrate.

The Factor, in contrast to the first barrier mentioned, is affected by penicillin and by temperature. Perhaps of greatest interest here, however, is the fact that the magnitude of the Factor appears to be a function of the cellular  $\beta$ -galactosidase concentration and that its value appears to vary in a different manner in adapting and in deadapting cells (Fig. 1). In fully adapted cells the Factor has a value, under our conditions, of approximately fifteen, while in unadapted cells it is unity. Thus in fully adapted cells a major portion of the  $\beta$ -galactosidase appears to be masked, whereas in unadapted cells all of the enzyme is active. In the early stages of  $\beta$ -galactosidase adaptation the Factor rapidly changes from unity to near maximum values. In a deadapting culture, however, the Factor is approximately halved for each cell division. The observation that in fully adapted cells the bulk of the formed enzyme is unavailable to the substrate might reasonably suggest that the formed enzyme is bound or associated with some cellular component. The additional observation that the Factor behaves differently in adapting and deadapting cells may in turn provide some evidence concerning the role of such a component. In adapting cells the rate at which the



newly formed  $\beta$ -galactosidase becomes masked is maximal during the initial stage of induced  $\beta$ -galactosidase formation, although the proportion of unavailable enzyme continues to increase during adaptation. In deadapting cultures, however, the Factor decreases in a linear fashion with the concentration of the enzyme per individual cell. During the first divisions of a deadapting culture the

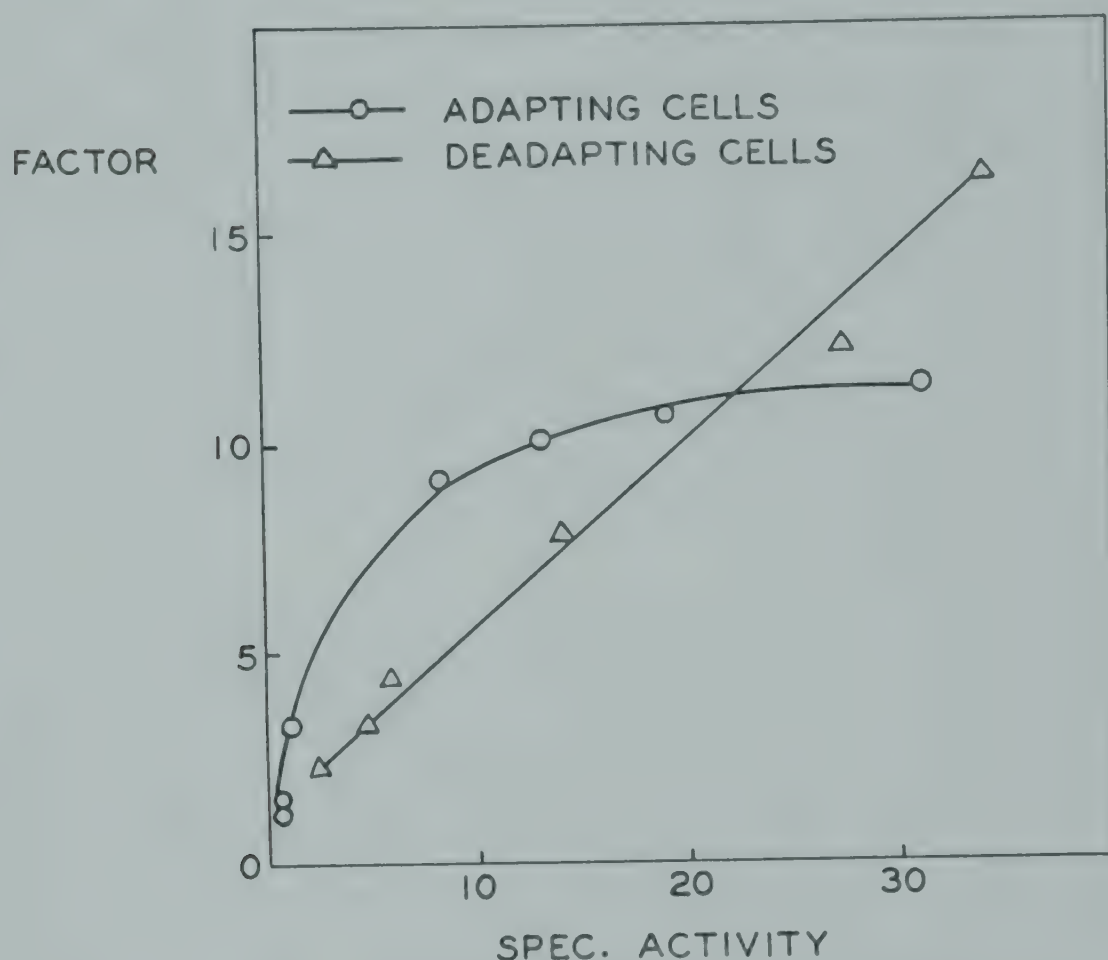


FIG. 1. The "Factor" as a function of specific  $\beta$ -galactosidase activity during adaptation and during deadadaptation.

amount of  $\beta$ -galactosidase available, in terms of intact-cell activity, approximately doubles for each cell division. Since a greater portion of the enzyme is masked during adaptation than during deadadaptation, and since the masking suggests that the enzyme is temporarily associated with some cellular component, it may be possible that this component is the still unidentified enzyme-forming system. To account for the inverse relationship between the factor and the  $\beta$ -galactosidase concentration of the individual cell one might visualize that, during the formation of  $\beta$ -galactosidase, enzyme molecules pile up much as shuttle-cocks are packed in cans. This would imply that

only the top or outer molecules would be fully accessible to the substrate. When the pack reaches a certain limiting height the topmost  $\beta$ -galactosidase molecule might spill over or become dissociated, with the result that more  $\beta$ -galactosidase then becomes available to the substrate. During deadaptation, cell division in the absence of  $\beta$ -galactosidase formation might lead to a doubling of the available enzyme by division or by halving the associated enzyme pack and thus might expose twice as much  $\beta$ -galactosidase to the substrate. Evidence of the sort described of course tells us little about the nature of the cellular component with which formed  $\beta$ -galactosidase is associated, other than perhaps to suggest that it is particulate.

One other point also deals with this Factor. As I have mentioned, unadapted cultures are found to have a factor of approximately one. By appropriate use of this observation, it can be shown that unadapted cells have a small amount of  $\beta$ -galactosidase and that this low  $\beta$ -galactosidase level of unadapted cells represents the homogeneous distribution of the enzyme throughout all the cells of the population. This observation is of considerable interest, since it shows that cells which are genetically capable of synthesizing  $\beta$ -galactosidase form a small amount of this enzyme even in the absence of an exogenous inducer.

In short, these observations suggest that overall protein formation is neither a prerequisite nor a necessary characteristic of the formation of this specific enzyme. Additionally, these observations strongly emphasize the importance of organizational factors during enzyme formation.  $\beta$ -galactosidase certainly is not formed in such a way that each enzyme molecule becomes active. Rather, the enzyme appears to be associated with some structure in the cell, and this structure may well prove to be a necessary and integral component of the  $\beta$ -galactosidase-forming system.

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# THE REVERSIBLE ACTIVATION OF ADAPTIVE ENZYMES IN THE ABSENCE OF SUBSTRATE BY A VITAMIN B<sub>12</sub> SYSTEM

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OGINSKY et al. (1) have shown that vitamin B<sub>12</sub> stimulates the oxidation of many substances in aged cells of *Escherichia coli* mutant 113-3. Bartron and I have presented evidence that vitamin B<sub>12</sub> activates individual enzymes of this mutant and not common intermediates in the oxidative pathway (2, 3). Many non-oxidative enzymes are similarly stimulated. Vitamin B<sub>12</sub> alone is often ineffective and must be supplemented by glutathione and glucose-6-phosphate.

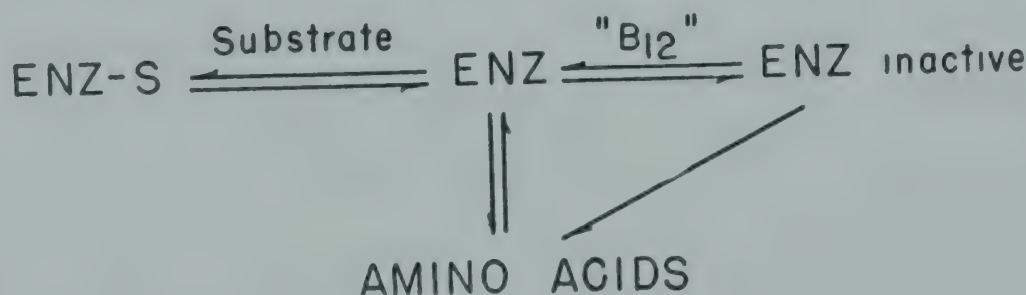


FIG. 1.

These studies on the reactivation of constitutive enzymes suggested that adaptive enzymes might not be detectable under the usual methods of assay because of their rapid inactivation. The successful reactivation of several adaptive enzymes in the absence of the substrates led to the simple scheme shown in Fig. 1. This scheme assumes that the organism synthesizes all the enzymes within its genetic capabilities. In the presence of substrate each enzyme is stabilized by the formation of an enzyme-substrate complex. In its absence the enzyme is reversibly inactivated. This instability in the absence of substrate may be the basic characteristic of adaptive

enzymes as a group. It accounts for the phenomenon of deadaptation as well.

The formic hydrogenlyase system is well suited to these studies, for its activity can be measured in the presence of the  $B_{12}$  system and adaptation to this system is not observed in washed resting cells. *E. coli* cells grown on casein produce formate and contain hydrogenlyase. The solid lines in Fig. 2 show the evolution of gas after

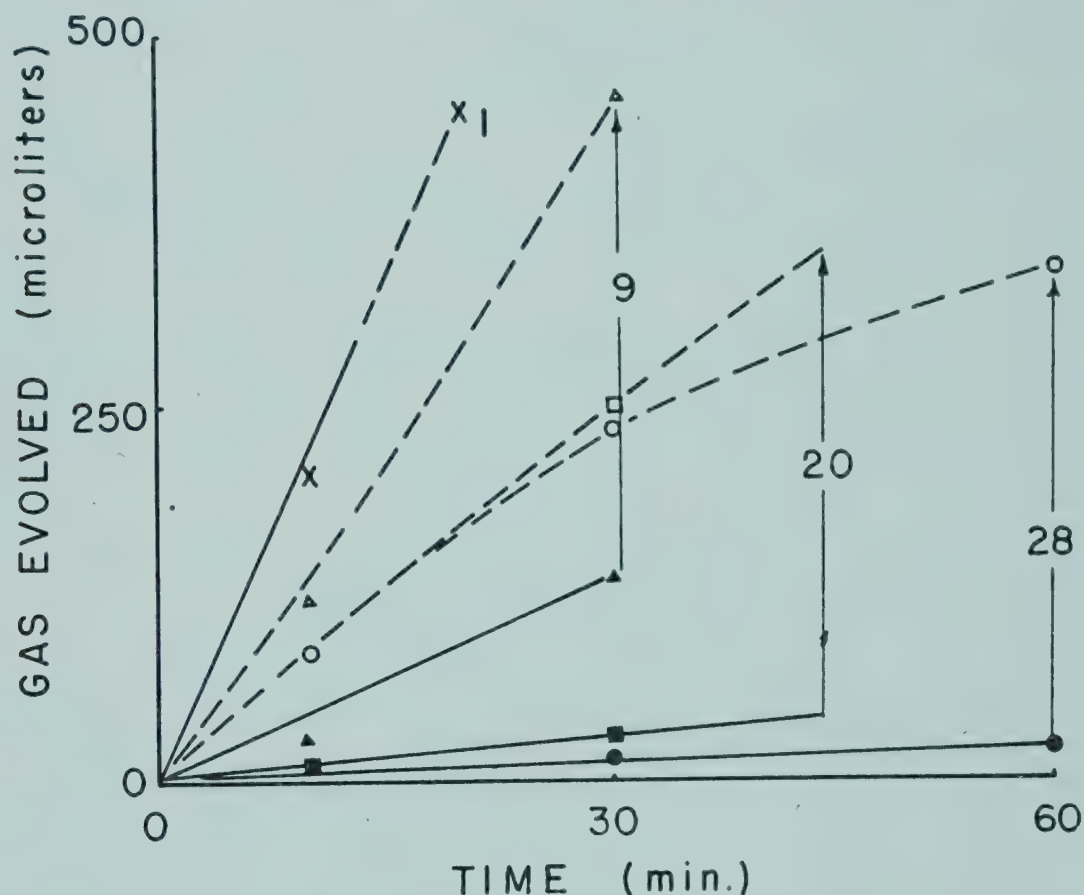


FIG. 2. Inactivation and reactivation of formic hydrogenlyase in *E. coli* cells grown on casein by the  $B_{12}$  system.

Solid curves give the rate of evolution of  $CO_2$  and  $H_2$  from lithium formate after aging of cells for the days indicated by the number on the vertical arrow at the end of each curve.

The dotted curve at the end of each arrow gives the gas evolution of the corresponding  $B_{12}$ -activated cells. Gas evolution in the absence of substrate has been subtracted from each curve in this and all the following graphs.

aging such cells at  $4^\circ C$ . for the respective numbers of days indicated on the graph. If the cells are treated with 50 mg. of  $B_{12}$ , 50 $\gamma$  glutathione, and 2 mg. of glucose-6-phosphate, the rate of gas production is increased by the amounts shown by the dotted curves. Freshly harvested cells are not stimulated by the  $B_{12}$  system. After 28 days most of the hydrogenlyase activity disappears, but can be



partially recovered by treatment for ten minutes with the B<sub>12</sub> system. Similar results have been observed with ornithine and lysine decarboxylase.

TABLE 1  
STABILIZATION OF HYDROGENLYASE BY FORMATE

Time (days)	Unstabilized	Formate stabilized
0	255 $\mu$ l./hr.	255 $\mu$ l./hr.
1	160	250
3	75	232
7	32 (210)	112 (400)

These bacteria had aged 10 days before formate was added at time 0.

Table 1 shows that the slow inactivation of hydrogenlyase can be retarded by the addition of formate to the cell suspension. The figures in parentheses give the degree of reactivation attainable in each case by "B<sub>12</sub>" treatment. It should be noted that a considerable part of the enzyme is irreversibly inactivated.

No hydrogenlyase is observed when the mutant 113-3 is grown on methionine and glucose. In this case formate often inhibits the evolution of gas (Fig. 3, lower curve). After activation by the B<sub>12</sub> system a constant rate of evolution of gas from formate is observed (open circles). This curve is a straight line passing through the origin and demonstrates that the enzyme has been formed in the absence of substrate. If the bacteria are treated and the activation system removed by centrifugation 15 minutes before the addition of formate, the activity falls to the values indicated by the squares. If the addition is delayed 30 minutes, no net evolution of gas is observed (curve marked by triangle). This rapid loss of activity is a measure of the instability of the enzyme in the absence of formate. This very high rate of inactivation of the enzyme which has never been exposed to formate may be contrasted with the greater stability of the enzyme which has been exposed to formate during growth and which presumably contains residual amounts of the substrate (Fig. 1). The stability after removal of the activating system is also much greater in the latter case.

Maltase is reasonably stable even after removal of the activating system (Fig. 4, upper curve). Activity is high at the time of addition of maltose, and there is no induction period. The pretreatment of

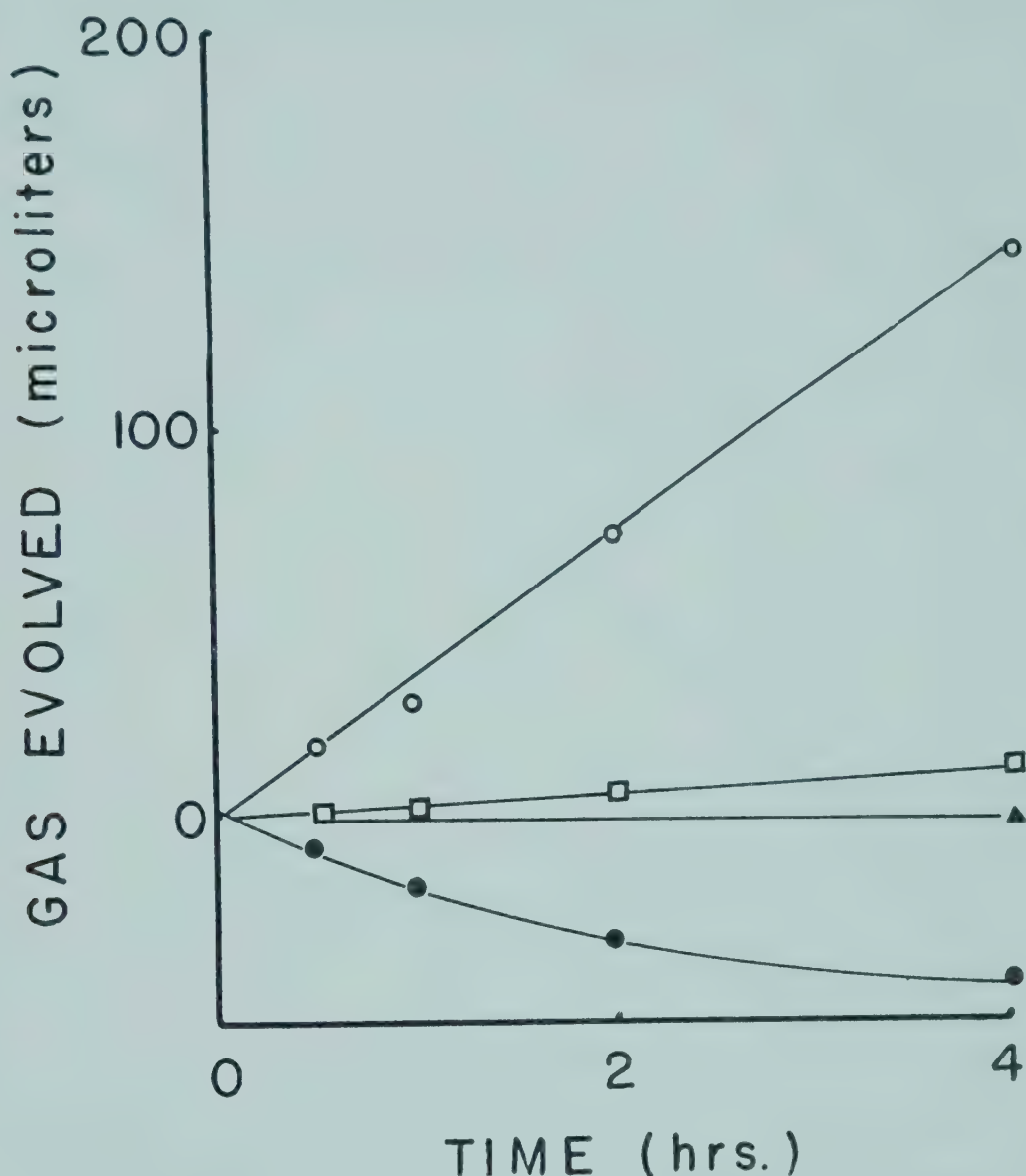


FIG. 3. Evolution of  $\text{CO}_2 + \text{H}_2$  from formate by the formic hydrogenlyase system in *E. coli* mutant 113-3 grown on methionine.

Solid circles: unactivated cells.

Open circles: cells reactivated with  $\text{B}_{12}$ , GSH, and glucose-6-phosphate. These compounds present throughout experiment.

Squares: cells reactivated by the  $\text{B}_{12}$  system, then separated by centrifugation. Formate added 15 minutes later at 0 time.

Triangle: cells reactivated by the  $\text{B}_{12}$  system, then separated by centrifugation. Formate added 30 minutes later.

15 minutes required in the absence of substrate is small compared with the 1-hour induction period before synthesis of new enzyme in the presence of maltose (lower curve).



Adaptation to lactose is more rapid in cells of the same strain (lower curve, Fig. 5). The upper curve, representing reactivated cells, is a composite of the activities of preformed activated enzyme and the newly synthesized enzyme. If the lower curve is subtracted

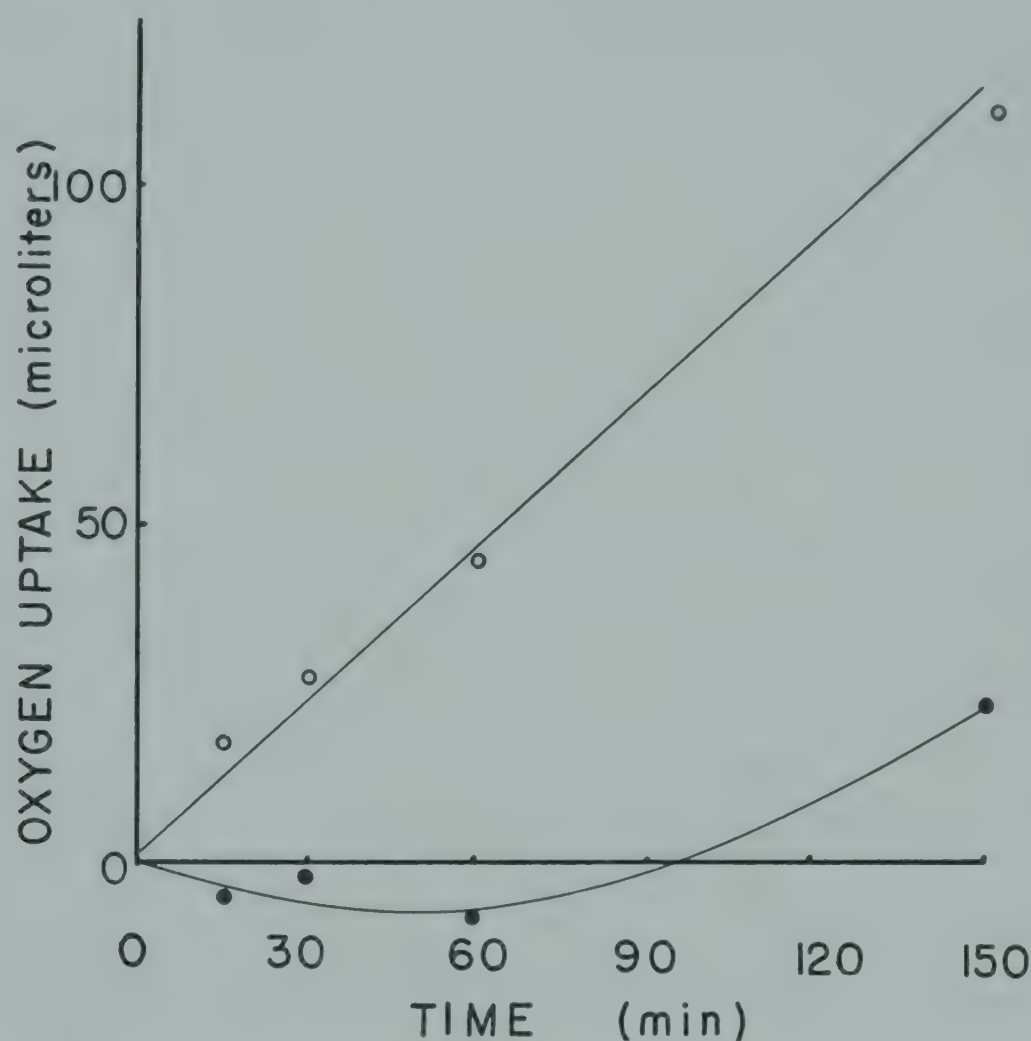


FIG. 4. Activation of maltase in *E. coli* 113-3 grown on methionine.

Lower curve: Maltase activity in untreated cells.

Upper curve: Maltase activity in cells treated with the B<sub>12</sub> system. Maltose added 1/2 hour after removal of activating system.

from the upper curve, the oxygen uptake of the preformed enzyme is obtained (dotted line). This is a straight line passing through the origin.

No  $\beta$ -galactosidase adaptation is observed in extracts obtained by alumina grinding. However, an increase in activity of the  $\beta$ -galactosidase is observed after a short pretreatment of the extract with the B<sub>12</sub> system (Table 2).

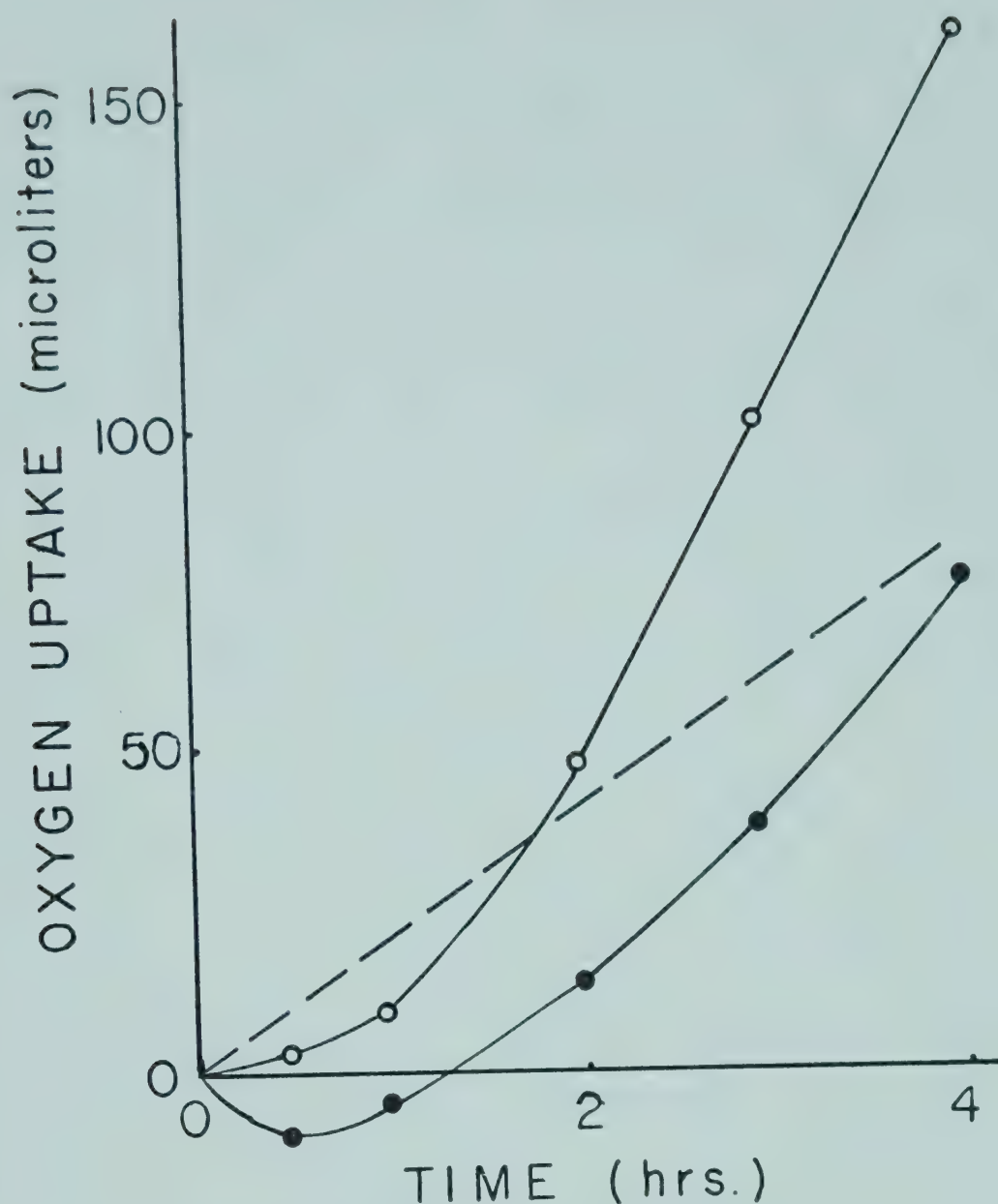


FIG. 5. Activation of lactase in mutant 113-3.

Closed circles: Lactose oxidation in unactivated cells.

Open circles: Lactose oxidation activated by 15 minutes treatment with the B<sub>12</sub> system. Lactose added at 0 time 15 minutes after removal of the activating system by centrifugation.

Dotted line: difference between oxygen uptake in reactivated cells and unactivated cells.

TABLE 2

 $\beta$ -GALACTOSIDASE ACTIVITY IN *E. coli* 113-3

	Increase in density at 410 m $\mu$ /40 min.
Control cells	.055
Treated cells	.195

6 g. of wet cells were disrupted by alumina grinding, extracted with 18 ml. water, and centrifuged at 12,000 g. 0.3 ml. of the extract was activated with the B<sub>12</sub> system in a total of 2 ml. for 15 min. The activating system, which interferes with the enzyme determination, was removed by dialysis against 0.001% lactose solution. The control was similarly treated. 0.5 ml. of ortho-nitrophenyl- $\beta$ -galactoside were added to the dialyzed samples.



## DISCUSSION

The data show that so-called adaptive enzymes exist in an inactive form and can be activated under conditions that do not permit protein synthesis. Since these enzymes can be formed in quantity in the absence of substrate, there is no need to postulate that the substrate acts as an inducer. Enzyme in the presence of substrate is simply accounted for by the well-known phenomenon of enzyme stabilization. Such a role is suggested by the studies on nitrase by Wainwright (3) and by Spiegelman and Reiner (4) on galactozymase. Enzyme stabilization may be achieved by non-substrate compounds which can reversibly combine with and protect active centers of the enzyme. Inhibitors should also be effective, provided their affinity for the enzyme is not so great that the presence of the enzyme cannot be detected. This mechanism accounts for the data on galactoside (5) and benzoic acid (6) analogues with less strain than the inducer-organizer theories, which become more complex as new data accumulate. The elimination of the substrate as an inducer makes the mass action hypothesis of Yudkin (7, 8) more acceptable as a mechanism for controlling enzyme concentration.

In view of the data presented here, the excellent evidence obtained by Pollack (9) for an induction effect by penicillin must be carefully reevaluated. The following points should be considered. The assumption that small amounts of substrate would be completely destroyed by the enzyme and, therefore, would be unable to maintain an enzyme-substrate complex is probably unwarranted. Neither affinity constants nor rates of reactions as ordinarily determined can be applied when the concentration of enzyme and substrate are equal (10). The present experiments suggest that the enzyme which has been exposed to substrate is more stable, even though the cell has been washed, than the reactivated enzyme which has never been exposed to substrate. This strongly suggests that the substrate is not completely destroyed. There may be some doubt that the concen-



tration of enzyme within the intact cell can be accurately determined from the turnover rate of the pure enzyme. If the enzyme is more effective within the cell than in the pure form the concentrations of penicillin fixed in Pollack's experiments may be sufficient to bind and stabilize all the enzyme formed.

The lack of demonstrable enzyme activity in the absence of substrate is a reflection of the relative instability of the enzyme. This also accounts for the phenomenon of deadaptation. Whether the inactive form of an enzyme can be reactivated in a given instance depends in turn on its own stability. The data presented suggest that the inactive enzyme is partially and irreversibly degraded, possibly to amino acids, as shown in Fig. 1.

The proposed theory, like the unitary theory of Monod (5), assumes that both constitutive and adaptive enzymes are synthesized by a similar mechanism, and accounts for the disappearance of constitutive enzymes (11) and simultaneous adaptation (12).

The view that enzymes are continuously synthesized in the absence of substrate finds support in the work on the  $P_z$  protein described by Monod and coworkers (13, 14). They found that all organisms capable of adapting to  $\beta$ -galactosides contain this immunologically related protein, which is not a precursor of the galactosidase but decreases under conditions of adaptation. This inactive protein has been an anomaly in other adaptation theories. It is required in the present theory and undoubtedly corresponds to the inactive enzyme.

Whether this simple theory can account for all the facts remains to be determined. The data raise doubt as to the validity of any theory requiring the substrate as an integral part of the mechanism of enzyme synthesis.

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# THE AUTOCATALYTIC PRODUCTION OF TYROSINASE IN EXTRACTS OF *DROSOPHILA MELANOGASTER*<sup>1</sup>

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THE EXPERIMENTS on the tyrosinase of wild-type *Drosophila melanogaster* which are to be described were undertaken as a preliminary to an investigation of tyrosinase genetics in this species. Our interest in the latter subject was initiated by the results of a recent study in which it was found that the thermostability of the tyrosinase of *Neurospora crassa* is inherited in a simple way (1). In order to analyse further the relationship between individual genes and the specific properties of the enzyme it is desirable to employ an organism in which a variety of mutations affecting the melanin-producing system is already known. This condition is fulfilled by *D. melanogaster*, in which a large number of body-color mutants has been found in the course of the long history of this species as a genetic organism. Although attempts by previous workers (2, 3) to relate the phenotypes of body-color mutants of *Drosophila* to the level of tyrosinase activity have been unsuccessful, it is clear that these investigators were hampered by inadequate methods both in the preparation of the enzyme and in the measurement of its activity. A reinvestigation of the problem therefore seems justified. The present results, although still preliminary in character, show that the tyrosinase system of *Drosophila* is considerably more complex than had been previously supposed.

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Shortly after this study had begun, we received Ohnishi's account of his experiments on *Drosophila* tyrosinase (4). Our results confirm and extend his finding that the enzyme occurs in an inactive form which becomes active on standing. Further comment on Ohnishi's paper will be found in the Discussion.

### EXPERIMENTAL PROCEDURES

The experiments to be described were carried out with adult flies of the Canton-S stock. Adults were used in preference to earlier stages because of the ease with which they can be collected. In this respect our experiments differ from those of the previously cited workers, all of whom employed larvae or pupae—possibly because of Graubard's report that he had been unable to extract tyrosinase from adult flies (2). We have experienced no difficulty in obtaining high activities from adults.

Most of the experiments were performed with six-hour flies. (Collections so designated actually consist of flies of various ages up to six hours from emergence). Tests carried out on older flies have shown that there is little change in the amount of extractable tyrosinase during the first 24 hours, but two-day-old flies yield only one-half, and ten-day-old flies one-tenth, as much activity as do newly emerged adults. After they were collected, the flies were stored at  $-25^{\circ}\text{C}$ . until needed. Extractions were made by grinding with 20 parts of cold 0.1 *M* phosphate buffer, pH 6, followed by centrifugation for 5 minutes at 20,000 *g* in a Spinco refrigerated ultracentrifuge. The supernatant, consisting of a clear aqueous layer and a thin lipid layer, was decanted and used as the source of enzyme. The entire preparative procedure takes less than 15 minutes.

Tyrosinase determinations were carried out by the colorimetric method (1, 5). The assay system consisted of 0.1 ml. of *Drosophila* extract, 3.9 ml. of 0.1 *M* phosphate, pH 6, and 1 ml. of 0.02 *M* L-3, 4-dihydroxyphenylalanine. In some experiments 0.75 ml. of 0.0167 *M* L-tyrosine was used as substrate. In either case, the indicated concentrations are saturating for the *Drosophila* enzyme. The maximal rate of dopachrome formation at  $30^{\circ}\text{C}$ . (identical with

the initial rate when dopa is the substrate, but not when the substrate is tyrosine) is proportional to the enzyme concentration.

### THE KINETICS OF ACTIVATION

Fresh extracts of adult *Drosophila* are devoid of tyrosinase activity, but they become active on standing at 0° C. This interesting process will be called "activation." In preliminary experiments it was found that activation is inhibited by dilution of the extracts, a fact sug-

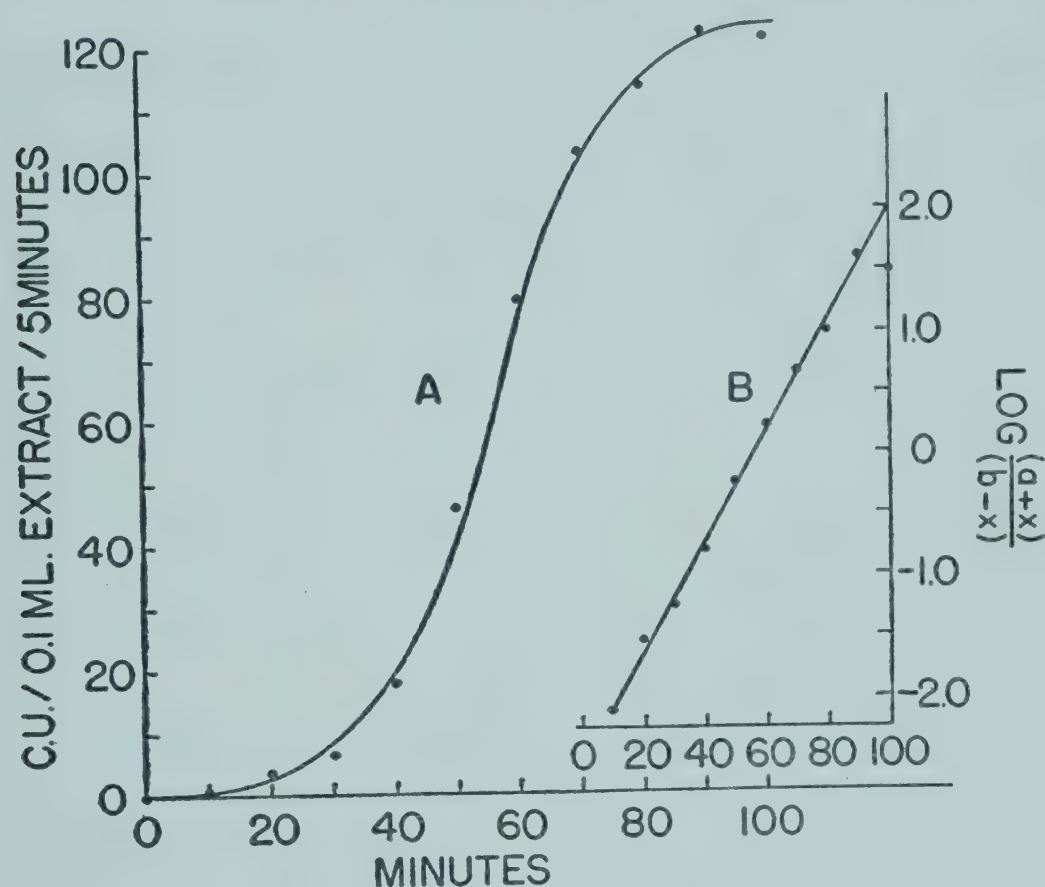


FIG. 1. A. Tyrosinase activity in a *Drosophila* extract as a function of time. The curve is the theoretical for an autocatalytic process. C. u. = colorimeter units. B. The same data plotted in a linear form. See text.

gesting (a) that activation does not consist of the destruction of a dissociable inhibitor of tyrosinase, and (b) that the process depends on a bimolecular collision. The correctness of the second inference was shown by the results of a study of the kinetics of activation. The procedure was to take 0.1 ml. samples periodically from an extract kept at 0° C. and immediately to dilute them to 4 ml. with buffer in a colorimeter tube, so as effectively to halt activation. The tyrosinase activity was then assayed by the addition of substrate. The



results of such an experiment are shown in Fig. 1A. The curve closely approximates that of an autocatalytic reaction of the type  $A + B \rightarrow 2A + C$ , where  $A$  is the catalyst and  $B$  is its precursor. The closeness of fit can best be seen in Fig. 1B, where the integrated rate equation for an autocatalytic process,

$$k't = \frac{1}{a+b} \ln \frac{b(a+x)}{a(b-x)}$$

is plotted with  $\log \frac{(a+x)}{(b-x)}$  as ordinate. In this expression  $a$  is the initial concentration of  $A$ ,  $b$  the initial concentration of  $B$ , and  $x$  the amount of  $B$  reacted in  $t$  minutes. The term  $(a+x)$  is equal to the amount of tyrosinase activity at time  $t$ ; the term  $(b-x)$  is obtained by subtracting  $(a+x)$  from  $(a+b)$ , the level of tyrosinase activity finally attained.

When activation is allowed to take place at 25° C. there is a marked shortening of the lag period and some increase in the activation rate, but the level of tyrosinase activity finally attained is only about one-half that found at 0° C. Since the enzyme itself is relatively stable at room temperature, this result suggests that the activating system undergoes destruction at 25° C.

The activation phenomenon is observed with respect to the oxidation of both tyrosine and dopa. On the basis of this test and also in view of the fact that we have not been able to separate the two activities on the basis of thermostability, it appears likely that this system does not contain a dopa oxidase separate and distinct from the tyrosinase.

#### THE ACTIVATOR

The kinetic findings suggest a model of the trypsin-trypsinogen type, in which tyrosinase is formed from a precursor by the tyrosinase-catalyzed oxidation of one or more phenolic groups in the precursor. This hypothesis was tested in a series of experiments in which the activating system was subjected to conditions under which tyrosinase is inactive. The conditions tested included the absence of oxygen (which was displaced by argon) and the presence of inhibi-

tors and substrates of tyrosinase. Use was again made of the fact that activation can be halted at any time by diluting the extract with buffer. Inhibitors were used in the minimum concentration necessary to produce complete or nearly complete inhibition of tyrosinase activity. The 50-fold dilution of the assay sample served to reduce the inhibitor concentration to a point where its effect on the tyrosinase assay was negligible. In the cases of cysteine, diethyldithiocarbamate, and cyanide, however, it was found that relatively stable complexes are formed with the enzyme, of such a nature that complete dissociation does not occur immediately following dilution. Special controls were set up to correct for this effect.

The substrates tyrosine and dopa were tested on the assumption that they would behave as competitive inhibitors of activation if the latter were tyrosinase-catalysed. In addition, a number of metabolic poisons were tested in an attempt further to elucidate the nature of the activation reaction.

TABLE 1

THE EFFECT OF INHIBITORS ON THE ACTIVATION OF *Drosophila* TYROSINASE

Inhibitor	Concentration	Percent inhibition	
		of tyrosinase	of activation
Absence of oxygen		100	0
Copper-binding agents:			
Cysteine	0.001 M	100	28
Diethyldithiocarbamate	0.00004 M	100	0
Cyanide	0.001 M	100	0
Azide	0.01 M	84	14
Competitive inhibitors:			
3-Aminotyrosine	0.001 M	78	0
Toluhydroquinone	0.001 M	88	< 10
Substrates:			
L-Tyrosine	sat'd.		0
L-Dopa	0.004 M		< 10
Metabolic inhibitors:			
Iodoacetamide	0.001 M		10
Fluoride	0.005 M		< 10
2, 4-Dinitrophenol	0.001 M		24
Arsenate	0.01 M		< 10
Arsenite	0.001 M		< 10



The results of these experiments are summarized in Table 1. They show clearly that inhibition of activation does not parallel inhibition of tyrosinase activity. It appears that that part of the tyrosinase molecule which is involved in the oxidation of phenols—including the copper atom and the site of substrate attachment—does not participate in the activation reaction. In fact, it is very unlikely that the activation process involves an oxidation of any kind. The results thus contradict the hypothesis stated above. The possibility is not excluded, however, that activation is catalyzed by a region of the tyrosinase molecule not concerned with the oxidation of phenols.

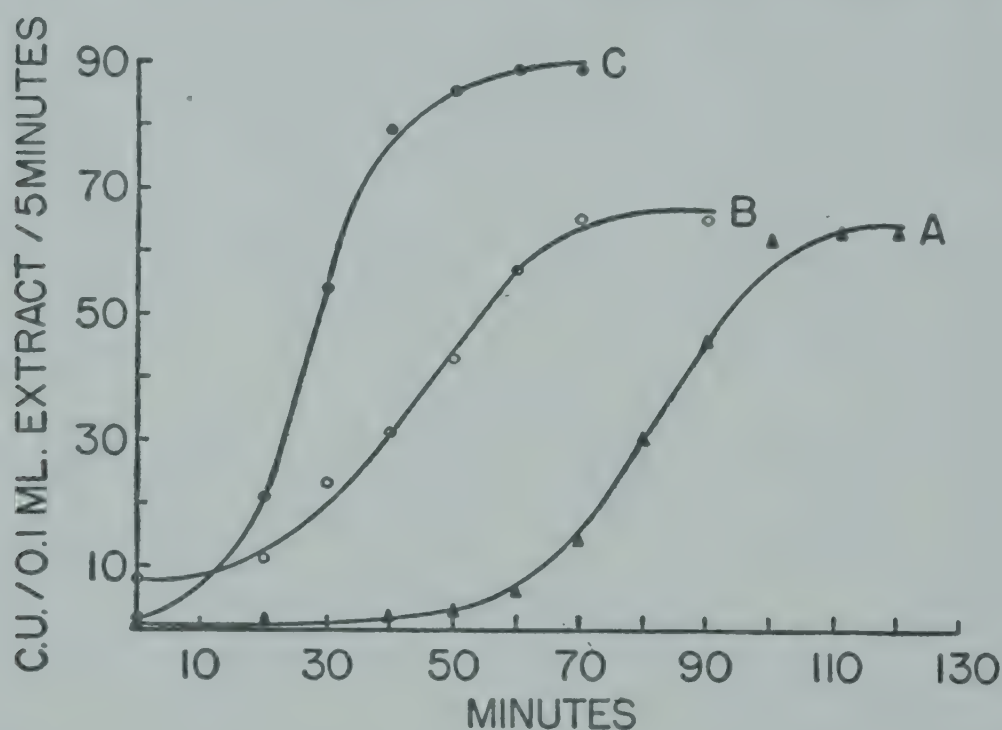


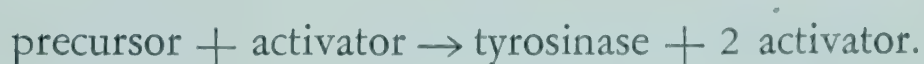
FIG. 2. A. The increase in tyrosinase activity in a *Drosophila* extract. B. The same, plus active tyrosinase. C. The same as A, plus the supernatant from an activated extract.

This question was answered by experiments in which active tyrosinase was added to fresh extracts.

In these experiments, advantage was taken of the fact that the enzyme tends to aggregate after activation. Seventy to eighty per cent of the tyrosinase activity can be spun out of a fully activated preparation by centrifuging at 40,000 *g* for ten minutes. The precipitate can be washed and resuspended without loss of activity. The effect on the rate of tyrosinase appearance of mixing such a preparation with a fresh extract is shown in Fig. 2, curve B. There is a shortening of the lag, but no perceptible increase in the slope of

the curve. This result suggests that the added tyrosinase does not function as activator, but that it is contaminated with a trace of activator. On the other hand, when the supernatant from which most of the tyrosinase has been removed is added to the extract a marked increase in the rate of activation is noted (Fig. 2, curve C). It is thus evident that the activator of tyrosinase is not tyrosinase itself, but a substance which is separable from it and the concentration of which also increases during activation. Preliminary experiments have shown that the activator is non-dialysable and is precipitated by 35 per cent saturated ammonium sulfate.

These results can be most simply accounted for by the following model:



According to this hypothesis, tyrosinase is a product of an autocatalytic reaction in which it takes no part.

No convincing explanation has yet been found for the fact, clearly seen in Fig. 2, that the addition of activator increases the final yield of tyrosinase activity. The example shown in the figure is an extreme case of this effect; in other experiments the extra yield has been of the order of 15 per cent. A possible explanation is based on the assumption that the precursor is subject to destructive action in the extract; addition of activator, by eliminating the lag, would increase the yield of active enzyme. Further studies of the effect are required before this hypothesis can be accepted.

#### P<sub>H</sub> DEPENDENCE OF ACTIVATION

The p<sub>H</sub> dependence of the activation reaction has been studied with the use of phosphate buffers in the p<sub>H</sub> range 5.3 to 7.6. Fresh extracts adjusted to various p<sub>H</sub> values were allowed to activate at 0° C. for 2.5 hours, and their tyrosinase activity was then measured. The values so obtained are a measure of activation rates, since if activation is allowed to proceed for longer periods of time the region of the curve in the neighborhood of the p<sub>H</sub> optimum tends to flatten out. At the extremes of the curve, however, there is probably



some irreversible inactivation; at pH 5 and pH 8.4 the activating system is largely destroyed.

Two main points emerge from the pH study: (a) the activation process is very sensitive to pH changes; and (b) the pH optimum depends on the method of extraction. In Fig. 3 are shown pH curves

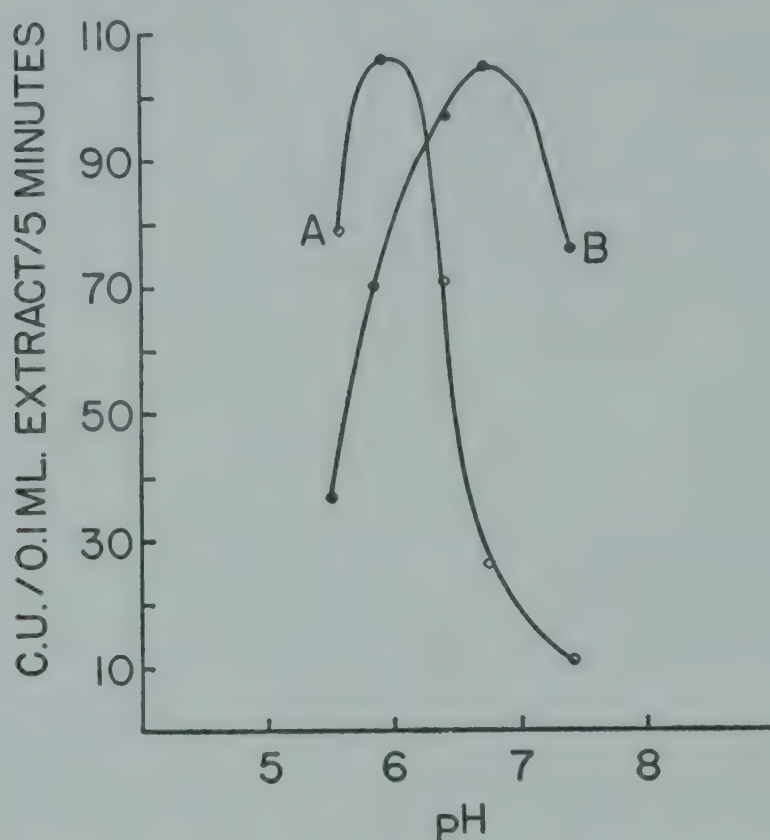


FIG. 3. A. Activation vs. pH in a water extract. B. The same in a saline extract.

of activation obtained, respectively, with extracts prepared in water and in 0.6 per cent sodium chloride. The pH optimum in the water extract is in the neighborhood of pH 6, whereas in the saline extract it is near pH 6.8. A number of experiments have been carried out in order to throw some light on this rather unexpected effect. It was established that the addition of sodium chloride to a water extract does not cause a shift in the optimum, which remains at pH 6; the effect is found only if the sodium chloride is present in the extracting medium. The possibility that different enzyme systems are extracted by the two solvents was tested by comparing the kinetics of activation in water and sodium chloride extracts at their respective pH optima, and by comparing the resulting tyrosinases with respect to their thermostability, pH optima for the oxidation of tyrosine and dopa, and relative rates of attack on tyrosine and dopa. No

significant differences were found in any of the tests, and it is therefore concluded that the same system is extracted in both cases. The remaining possibility is that one of the extracts contains a substance, not present in the other, which influences the pH optimum of activation. This hypothesis was confirmed by experiments in which the two kinds of extracts were mixed. The pH optimum in such mixtures is that of the sodium chloride component. The re-

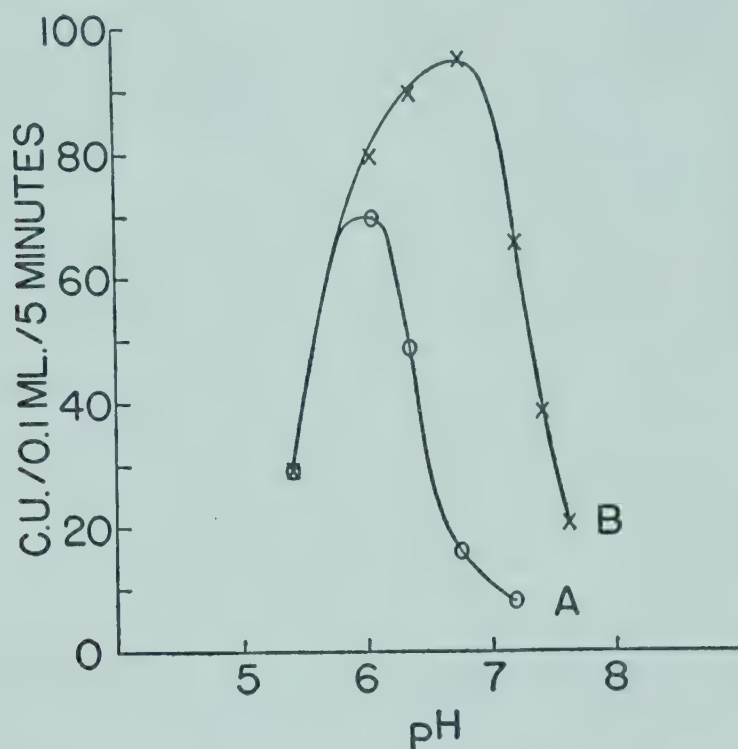


FIG. 4. A. Activation vs. pH in a water extract. B. The same, plus a tyrosinase-free saline extract of *Drosophila*.

sponsible factor in saline extracts can be obtained free of the tyrosinase system by extracting the flies first with water and then with saline. When added to a water extract, this preparation causes an increase in the activation rate at the higher pH values, resulting in a shift of the optimum from pH 6 to pH 6.8 (Fig. 4). The factor is not dialysable; it is precipitated by 50 per cent saturated ammonium sulfate. Its preparations do not spontaneously develop tyrosinase activity when allowed to stand at pH 6.8.

#### THE INHIBITOR OF TYROSINASE

Still another component of the *Drosophila* system is a tyrosinase inhibitor which can be detected in fresh extracts. This substance



is of interest in the present context because of the possibility that destruction of an inhibitor might account for the entire activation phenomenon. This possibility seems to be clearly excluded in the present instance by the relationship between tyrosinase activity and inhibitor concentration which is plotted in Fig. 5. The enzyme used in this experiment was a preparation of washed, active tyrosinase.

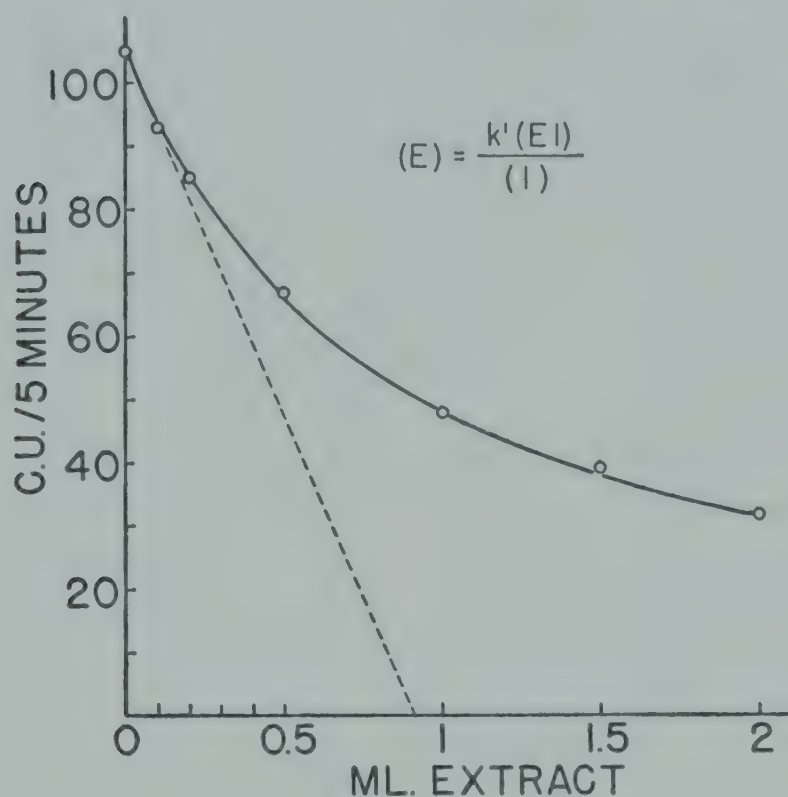
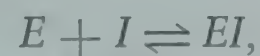


FIG. 5. Tyrosinase activity vs. concentration of the natural inhibitor in fresh extracts. The solid curve is that of the function shown and represents a simple reversible equilibrium reaction. The dotted line is that expected on the hypothesis of irreversible combination between enzyme and inhibitor.

The inhibitor was a fresh phosphate buffer extract of *Drosophila*. At the dilution employed, activation of the extract did not occur within the time of the test. The observed inhibition values of tyrosinase activity fall on the theoretical curve for a reversible enzyme-inhibitor reaction:



$$(E) = \frac{k'(EI)}{(I)}.$$

Since a saturating concentration of substrate is used in the assay, the observed tyrosinase activity is proportional to  $(E)$  in the above

expression.<sup>2</sup> Experiments in which the substrate concentration was varied have shown that the inhibition is independent of the substrate concentration and is therefore non-competitive. Separate experiments have also confirmed that the enzyme-inhibitor complex dissociates on dilution. In order to account for the fact that dilution of fresh extracts prevents activation, it would of course be necessary to postulate a non-dissociable compound between enzyme and inhibitor, a condition which is clearly not met here. The broken line in Fig. 5 shows the inhibition curve expected on the hypothesis of non-dissociable *EI*.

While it is thus excluded that the inhibition found here is responsible for the absence of tyrosinase activity in fresh extracts, it is not ruled out that the inhibitor plays some less obvious role in the activating system, nor is it likely that it is without significance in the biological process of melanic differentiation.

## DISCUSSION

The preliminary results on the *Drosophila* tyrosinase system which have been presented above show the system to be one of considerable complexity, although not more so than is indicated by genetic data. While nothing has been found which is irreconcilable with the hypothesis of tyrosinase formation via the modified autocatalytic process stated above, and while there is much in its favor, yet it is also true that there are a number of features about the system which are not yet understood. This, coupled with the fact that the proposed mechanism is very unusual, if not unique, among known biochemical processes, requires us to consider this interpretation as a working hypothesis only, pending further analysis of the activation process.

This report is not the first to deal with the activation of insect tyrosinase. The phenomenon has been found previously in extracts

<sup>2</sup> It can be seen in Fig. 5 that at a concentration of 0.1 ml. of extract in a volume of 5 ml.—the concentration normally employed for assays—there is an approximate 10 per cent inhibition of tyrosinase activity. This means that the enzyme activities reported in this paper are too low by this amount. This in no way affects the interpretation of the data.



of *Drosophila* larvae by Ohnishi (4) and in grasshopper embryos by Bodine et al. (6). On the basis of comparative microdissection experiments in water and saline, Ohnishi concludes that the body fluid contains an inactive form of tyrosinase and the tissues an activator. This hypothesis is not necessarily at variance with the one proposed above, but in view of the facts we have brought to light concerning the complexities of the *Drosophila* system, and in view of the inconclusive nature of some of the data on which Ohnishi's theory is based, we cannot regard it as established. According to the reports of Bodine and coworkers, grasshopper extracts contain a protyrosinase and an activator which can be separated by low-speed centrifugation, the activator going into the lipid layer. The protyrosinase is activated on mixing it either with the natural activator or with a variety of protein denaturing agents. We have attempted to reproduce these results in extracts of *Drosophila* without success. Stable preparations of the tyrosinase precursor have not been obtained by centrifugation, nor have we been able to detect any activating effect of sodium oleate, sodium lauryl sulfate, or urea, all of which are active in the grasshopper system. It appears that the *Drosophila* system differs at least superficially from that which has been studied in the grasshopper embryo.

We are indebted to Prof. E. B. Lewis for helpful discussions and advice on the handling of *Drosophila* cultures.

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## DISCUSSION

DR. JAKOBY: I wonder if Dr. Gale has tried using DNA from a mutant strain of his organism. If, for example, one were to use the DNA from a mutant unable to synthesize  $\beta$ -galactosidase and incubate it with the "poached-egg" system from a wild-type strain the resulting mixture would be unable to synthesize  $\beta$ -galactosidase. That is, it would be unable to synthesize the enzyme if the geneticist's present concept of the role of DNA were correct.

DR. GALE: No. We haven't tried it.

DR. DAVIS: I would like to say a few words about the question of turnover that Dr. Spiegelman raised. He reviewed briefly the isotopic experiments of Cohn and Hogness in Monod's lab, whose results were consistent with his own; and he graciously assigned to Dr. Monod a rather unique position in this field as prophet of the god  $\beta$ -galactosidase. I am sure Dr. Monod would modestly decline this compliment if he were here!

One of the main points that Dr. Spiegelman made is that after all this is only one enzyme system and so we have to be careful not to draw too far-reaching conclusions. I would agree with him; but at the same time I think it would be important not to just write off the whole thing and say that we can't draw any general conclusions from just one enzyme. Perhaps one can distinguish those conclusions pertaining to beta-galactosidase which may not pertain to other enzymes and those which necessarily will. Now, the most important aspect of that work, it seems to me, is that when you take a cell and label it with either radioactive sulfur or radioactive carbon and then remove it to a nonradioactive medium and induce the formation of beta-galactosidase, the beta-galactosidase which is being formed from amino acids coming in from the medium does not take up any extensive radioactivity from components of the cell already present. I think that this, then, tells us something about the cell that is not limited to the beta-galactosidase system—namely, that if there is any breakdown of various proteins already existing in the cell, this process cannot form free amino acids at a significant rate compared to the rate at which free amino acids are being taken in from the medium and incorporated into the proteins. It therefore seems to me that a very general conclusion can be drawn: that in the bacterial cell there is no turnover of proteins of the sort that one might have expected from an extrapolation of the earlier results on animal cells.

Dr. Spiegelman further mentioned two enzymes whose activity in "deadapting" cells decreased at a high rate, suggesting breakdown in addition to dilution—a different picture from that presented by beta-galactosidase. It seems to me that such disappearance of activity could be due to denatura-



tion and not necessarily to fragmentation of the enzyme molecules. However, such fragmentation, though not proved, might exist; and so the possibility must be considered that some other enzymes might differ from beta-galactosidase in being able to form such fragments and perhaps in being able to utilize them. Hence this formation and/or utilization of peptide fragments, which have been excluded for beta-galactosidase, have not necessarily been excluded for all other bacterial proteins. Nevertheless, the results already obtained with beta-galactosidase clearly show that such turnover of proteins, if present, is not contributing significantly to the amino acid pool; and this important general conclusion can be drawn without waiting for confirmation with other enzymes.

Another point that Dr. Spiegelman made—namely, that the time scale is very much shorter in bacterial than in animal cells—I would completely agree with. The difficulty would be to try to find what kind of parameter one should use to compare protein synthesis in bacteria with that in animal cells.

DR. BONNER: In this connection we have carried out some experiments on deadaptation following the adaptation of beta-galactosidase. In *E. coli* and on a population basis over a matter of about 7 divisions we could detect no loss of beta-galactosidase though per cell the concentration was halved for each division, thus no turnover was detected in seven generations though there may be turnover over long time periods which we could not detect.

DR. SPIEGELMAN: It was not my intent or desire to assign a position to anyone on the question of turnover. Rather I was concerned with evaluating the proper position of the experiment which was achieved in several laboratories by different methods. The data obtained are so impressively clear cut that it is admittedly difficult to avoid general conclusions. My point was, and still is, that any such extrapolations from the study of the formation of a single enzyme should be accepted with reservations. This is particularly true in view of our experience with yeasts indicating the existence of a mechanism leading to the internal replenishment of the free amino acid pool.

It might be noted that there is not only a matter of time scales to take into account in such experiments. There is also the question of the adequacy of the sampling size. In the course of performing a short term induction experiment, such as is necessary to decide the precursor question, only about .05 per cent of the proteins of the cell is being examined. One is therefore examining an exceedingly small fraction of the turnover, if it exists. The question also arises whether the enzyme being chosen is sufficiently representative of the other proteins of the cells. In this connection, it should be noted that the beta-galactosidase of *E. coli* possesses two properties which may or may not be relevant. It is a very large molecule, its molecular weight being in the neighborhood of 800,000. Furthermore, it is completely resistant to trypsin.



DR. KALLIO: I would like to mention some experiments which are in a very primitive form which may or may not have application to the subject under discussion here. The reason that I suspect that they may not is because we have been working with two aerobic organisms: *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* and in these organisms we have been unable at any time to demonstrate any amino acid pool whatsoever. The system that we have been using is the adaptive or inductive form of oxidation of benzoic acid by cells grown on either succinate or fumarate. These cells will oxidize benzoic acid after an appreciable lag, something of the order of 75 to 90 minutes. In other words, you get a typical adaptive response. If we starve these cells we do not abolish this effect; in fact the lag period is very appreciably shortened and the rate of adaptation is very considerably increased. Before or after starving we could not demonstrate an amino acid pool. Nevertheless, we can abolish the inductive synthesis of the enzyme system by incorporating amino acid analogues in the incubation mixture, and this inhibition is specifically reversed by the homologous amino acid. Like Dr. Spiegelman, we have been unable to find any peptides of any kind, large or small, in these cells. During the starvation period considerable amounts of ammonia are excreted by the cell. In addition to the ammonia which is excreted by the cell, a small amount of keto acid (primarily pyruvic acid) is also excreted. The amount of total keto acid excreted measured as pyruvic amounts to 25 to 30 per cent of the ammonia which is excreted. There is no remarkable change in either the RNA or the DNA following these starvation periods, which are of the order of 6 hours.

DR. SPIEGELMAN: Couldn't that mixture of keto acids and ammonia simply be your free amino acid pool?

DR. KALLIO: I think so, yes, if you will append to the scheme a couple of reversible arrows between keto acids and amino acids.

DR. HALVORSON: I have several observations which agree with some of Dr. Gale's comments. We have been interested in trying to separate DNA and RNA by ultraviolet light absorption similar to some of the experiments that Dr. Kelner has carried out in *E. coli*. We have found that dosages which would inhibit DNA almost completely would allow appreciable RNA and enzyme synthesis to continue, and this was further verified by following  $P^{32}$  incorporation in the nucleotides of RNA. If the yeast cells are instead irradiated at intervals after the addition of the inductor, we found that the system which formed enzyme became more resistant to ultraviolet light. Dosages of ultraviolet light which would completely inhibit an unadapted cell from increasing its enzyme captivity, only slightly retarded induced enzyme synthesis of a partially induced cell. The rate of subsequent enzyme formation appeared in these cells to be related to the prior exposure to the inductor. Although a constant rate of enzyme synthesis was maintained in



these irradiated cells, full enzyme capacity was not attained. After 30 minutes or so enzyme synthesis ceased, the level obtained being related to the time of irradiation. These observations are understood if we assume that an unstable type of RNA molecule was involved in the enzyme-forming system, its concentration depending upon the prior exposure to the inductor.

DR. STEINBERG: I would just like to call the attention of the group to another type of data which are concerned with the pathway between amino acids and proteins. In Dr. Anfinsen's laboratory we have been working with pure proteins instead of studying the total proteins: In these experiments we have started with labelled precursor amino acid and isolated the pure crystalline protein and degraded it to isolate amino acid residues from different portions of the same molecule, hoping in this way to get some clue as to what the pathway of that labelled precursor amino acid was on its way to the final protein form. We studied ovalbumin and have now studied 5 different amino acid residues of ovalbumin: glutamic acid, aspartic acid, alanine, glycine, and serine, and have been able to show that, for example, the residues of serine in the ovalbumin molecules synthesized *in vitro* have different specific activities. Similar results have been obtained for the other four amino acids mentioned. This work has been extended in some very elegant experiments by Dr. Vaughan and Dr. Anfinsen on the synthesis of insulin *in vitro*, and here again when the insulin is recrystallized to constant specific activity and then degraded by Sanger's method and residues from known parts of the insulin molecule compared, again, the residues of glycine for example show different specific activities by a factor of 2 or more. The residues of glycine show differences both within a chain and between the 2 chains. They also studied ribonuclease and have found that the phenylalanine residues in ribonuclease have different specific activities. If this were a simple compound and you found carbon atoms of different specific activities, you would conclude that they had different pathways in coming to the compound. We feel that in a similar manner these differences rule out a *simultaneous* condensation of the amino acids to form the protein, because such a process should lead to amino acid residues of identical specific activities at all points in the protein chain. Now, how this relates to the other mechanisms that have been suggested we of course don't know in detail, but I would like to stress one thing—that there should not be a distinction drawn between a "template mechanism" of synthesis and a "stepwise mechanism" of synthesis. These are two different aspects of the process of protein fabrication. The template hypothesis deals with a structural hypothesis which determines the sequence. It can very well be a stepwise process—that is, even though you have a template of some type on which the amino acids are, if you will, adsorbed from the medium, this can occur in a series of steps. It need not occur by simultaneous condensation of all



of them. Dr. Dalglish has published a mechanism of this kind in which he visualizes just such a process. If this were the process, it turns out that if there were any significant time necessary to fabricate the protein molecule you would observe the specific activity relationships which we have observed in our experiments on ovalbumin. That is to say, the degree of nonuniformity of labelling decreases with the length of time of incubation. At one and one-half hours specific activity ratio between one residue that we have isolated specifically and the average is 2.8, but at four hours it has fallen to about 1.4. Dr. Richard Hendler has done a larger series of experiments of this kind, and they confirm that the specific activity ratios decrease with time. This mechanism or a number of other mechanisms of stepwise synthesis, whether on a template or not are compatible with our results. Whether the intermediates are bound on a template and therefore precipitate with the trichloroacetic acid insoluble material or not, doesn't change the kinetic consideration on which we hope that this kind of approach may shed some light. I would like in closing just to point out that the pathway between amino acids and the enzyme based on Dr. Spiegelman's experiments rules out any other *ultimate* precursor but don't tell us what the length of this pathway may be or any of the intimate details of what that pathway may be.

DR. BEN-ISHAI: The work of Dr. Gale and of our group indicates that RNA synthesis has to occur for induced enzyme synthesis to take place. Dr. Gale has presented some evidence to the effect that DNA may also be involved in protein synthesis. However, if DNA is involved in protein synthesis, the results with the thymineless *E. coli* mutant, and also preliminary experiments with azathymine show that no new synthesis of DNA is necessary for induced enzyme formation.

DR. SPIEGELMAN: I would like to make a comment about Doctor Steinberg's remarks. This point is that tracer incorporation studies can in principle tell us two things about proteins: (1) They can provide information about their synthesis, (2) They also supply data on the ease with which constituent amino acids can exchange in already existent protein molecules. As demonstrated very clearly by the work of Gale, these two are experimentally dissociable processes. Until one can estimate the extent to which exchange incorporations influence the quantitative details of such experiments, it will be difficult to evaluate their relevance to the problem of the mechanism of synthesis of proteins.

DR. MAGASANIK: I would like to comment here on the phenomenon of deadaptation—the exposure of cells which are adapted—to a medium which contains a source of energy and building blocks but no longer contains the inducing agent. Dr. Spiegelman has mentioned particular cases, in one of which the enzyme is diluted out—that is, the enzyme is stable, but no more is formed. Another is one in which the enzyme is unstable. There is the



third case, of course, which was shown by Pollock, in which the enzyme continues to be formed at a linear rate. I would like to cite two observations on work which was done in our laboratory in collaboration with Mr. Neidhardt, in which we find that in *Aerobacter aerogenes*, using as our adaptive enzyme inositol dehydrogenase, we could show that cells adapted to inositol when placed in a medium free of inositol but containing histidine as an energy source would continue to make this enzyme, but when placed in a medium containing glucose as the energy source did no longer make the enzyme, although the enzyme was stable and was diluted out in due course similarly to the beta-galactosidase.

DR. SPIEGELMAN: In view of the results obtained by tracers, I frankly fail to see how Dr. Dubnoff's activation experiments can be accepted as relevant to the question of precursor in the induced synthesis of enzymes. The tracer experiments demonstrated conclusively, that subsequent to the addition of inducer, virtually all the carbon and all the sulfur of the enzyme molecules being synthesized comes from newly formed material.

DR. DUBNOFF: I don't think there is any contradiction here. I accept the results of your experiments showing that amino acids are taken up to form new enzyme in the presence of substrate. My experiments suggest that the substrate stabilizes the newly synthesized enzyme rather than actively inducing its synthesis. The inactive enzyme in my scheme is not formed nor is it necessarily reactivated under your conditions of active enzyme synthesis in the presence of substrate. The arrows in the diagram indicate potential pathways and should not be taken to mean that all the components are in rapid dynamic equilibrium under all conditions.

DR. SPIEGELMAN: If PZ is not converted into enzyme on the addition of the inducer, which by the way need not be a substrate to anything which combines with the enzyme, what role does PZ play . . . why postulate it as precursor?

DR. DUBNOFF: The reason for having it is simply that it is there. PZ has been demonstrated by immunological means although Monod has not been able to fit this compound into any theory of adaptation. I have been able to demonstrate the presence of this inactive enzyme by reactivating it. Since adaptive enzymes have been shown to be unstable in the absence of substrate the inactive form is in a sense a degradation product of the enzyme and not an intermediate.

DR. DAVIS: I would like to ask Dave Bonner whether in his system, in which he interpreted the difference in activity between the intact cell and the extract in terms of a form of the enzyme that was cryptic in the cell, it isn't equally possible that what you might have is the following situation: that the substrate can enter the cell at a slow rate; when there's very little



enzyme in that cell that rate is not limiting for the over-all reaction, so that the "factor" becomes 1; when you increase 15-fold the concentration of that enzyme in the cell, but the rate of permeation of the substrate into the cell hasn't increased in parallel, then that rate of permeation can be limiting for the reaction and you would have a high "factor." Is there anything in your evidence that would exclude that interpretation?

DR. BONNER: The "factor" is compensated for penetrability in a sense by using substrate concentrations which give maximal rates of hydrolysis, thus that aspect of it is already compensated for before the "factor" is determined.

DR. DAVIS: Do you believe that putting an excess of substrate in the medium really assures you that the rate of access of that substrate to the enzyme within the cell can no longer be rate-limiting for the reaction? If you have an active process of transport, then you could have a 1000-fold excess of substrate outside; yet something between the enzyme and the substrate itself could still be rate-limiting.

DR. GUNSALUS: I would subscribe to this view on the basis of some of the citrate-adapted systems. If you take *E. coli*, for example, no matter how long you grow it in the presence of citrate you can't get it to metabolize citrate at an appreciable rate in the intact cell. If you break up the cells, then the enzymes of the oxidative cycle are there and then your "factor" would become infinity.

DR. VOGEL: I would like to comment on this. I am not sure that the citrate system has relevance, because I don't think that you can in general get participation of exogenous citrate in the Krebs cycle of growing *E. coli* cells. This "factor" situation differs very materially, in that it is variable as a function of the adaptation of the organisms. Moreover there is no evidence of any kind for a specific transport mechanism for the substrate in this particular case, and, if there is one, it would remain to be shown that such a transport mechanism at the cell wall is dependent on the adaptive condition of the enzyme system under discussion. These considerations argue strongly against Dr. Davis' suggestion.

DR. BONNER: It is hard for me to account for why you have entirely different behavior of this system under adapting and deadapting conditions, if this is simply "permeability."

DR. DAVIS: Not at all. Under deadaptive circumstances you cut down at each cell generation the amount of enzyme to a half. If your cells still are all able to get the substrate in the same rate per unit of time per cell, the total fraction of your enzyme that can be active now doubles and the amount per cell halves—that is, you get something else that is rate-limiting.

DR. BONNER: What's rate-limiting?



DR. DAVIS: What's rate-limiting is the penetration of the substrate into the cell. If you have a half as many enzyme molecules per cell and a constant rate of penetration, you now get twice as much activity per enzyme molecule if the penetration is rate-limiting. It seems to me it's as simple as that.

DR. VOGEL: Now, I really can't agree with this. As mentioned by Dr. Bonner, adapting cells are characteristically different from deadapting cells as to change of the "factor" as a function of extract specific activity. In adapting cells there is initially a very rapid rate of change of the "factor," and then the "factor" remains substantially constant while the specific activity increases severalfold; deadapting cells, however, do not behave correspondingly. Consequently, as Dr. Bonner showed, it is possible to obtain cells having equal specific activity, but having different "factors" depending on the state of adaptation or deadadaptation of the cells. A simple scheme (such as suggested by Dr. Davis), which essentially depends only on variable specific enzyme activity and on constant, restrictive substrate penetration conditions, would predict equal "factors" for equal specific activities, and therefore obviously does not fit the data presented.

DR. BONNER: I fear that my presentation of "the Factor" was not crystal clear, and I would like to remention two aspects of this factor which I feel may clear up some of the apparent confusion. The "factor" is determined using substrate concentrations that permit maximal rates of substrate hydrolysis. One barrier, and we feel probably a permeability barrier, is thus compensated for in determining this factor. This of course in no way rules out permeability in accounting for the factor. The factor represents a barrier of some sort between the formed enzyme and substrate. The characteristics of this barrier, however, suggest that it is not a barrier involving cell membrane permeability, but rather reflects an intracellular organization in the formation of  $\beta$ -galactosidase. In the first place, the factor, is found to vary with the state of  $\beta$ -galactosidase adaptation of the cells. In unadapted cells all of the formed enzyme is accessible to the substrate, while in fully adapted cells only a small fraction of the formed enzyme is available to the substrate. Perhaps more important to the present discussion, however, is the fact that the factor varies differently in adapting and in deadapting cells. This difference can be seen most clearly in Fig. 1 of my paper in which "The Factor" is plotted as a function of the per cell  $\beta$ -galactosidase concentration in both adapting and deadapting cells. From this figure it can be seen that the amount of enzyme that is available to substrate differs in adapting and deadapting cells even though the total  $\beta$ -galactosidase content of both types of cells is equal. Thus the amount of enzyme available to substrate in cells of equal  $\beta$ -galactosidase content differs depending upon whether the cells have achieved this enzyme concentration by enzyme synthesis, i. e.



adapting cells, or by enzyme dilution, i. e. deadapting cells. It is for these reasons that we at present favor a "packet" interpretation of the barrier measured by the factor.

DR. GUNSALUS: I don't think we are going to settle this one right here.

DR. WOOD: I want to ask a question about the isotope experiment. If you have amino acids going to a precursor and then to an enzyme and you have precursor labeled with  $C^{14}$ -lactate, there may be only a smidgeon of precursor. It thus may have a small number of total counts but a high specific activity. Then you put in something, that increases enzyme formation, and increases the amino acids going into enzyme. It seems to me that you cannot exclude this possibility that there is precursor going into the enzyme until you measure accurately the amount of the precursor present. If the precursor had only 20 counts in it, then you are going to get only 20 counts into the final enzyme; so I don't think, at least as I understand the experiment, that it in any way excludes a precursor.

DR. SPIEGELMAN: I agree with you. We cannot categorically deny the existence of any performed precursor. I don't see how we will ever be in a position to make this statement. You are, of course, raising objections which are in general applicable to any conclusions of this nature. I might however note that in the best experiments we have, we can say that if there is a preexistent precursor, it can form less than 1 per cent of the carbon of the newly formed enzyme molecules. This 1 per cent is an upper limit, since the counts we were getting on the isolated enzyme were not detectably higher than the background. Furthermore, no difference is observed in the radioactivity found in the enzyme formed in short induction experiments, as compared with longer ones. In the former, the precursor should constitute a larger percentage of the enzyme being examined, and one might therefore expect to find higher radioactivities . . . even though they be at lower levels. No such difference, however, has been detected either in the  $C^{14}$  experiments or in the ones using  $S^{35}$ .

There are no experiments existent, to my knowledge, which permit one to say that a precursor cannot form less than 0.1 per cent of a newly formed enzyme molecule. However, on the basis of the data available, both with tracers and with amino acid analogues, I am not willing . . . at least at the present time, to design further experiments on the basis that a precursor does exist and plays a quantitatively significant role in the process of enzyme synthesis.

DR. GUNSALUS: You know the source of the enzyme but not its route. I believe Dr. Wood is primarily interested in the reaction route at the present time.

DR. SCHWEET: I gather that a very sharp distinction has been made today



between "exchange incorporation" and synthesis. I would like to suggest that much of the data presented is compatible with a mechanism of protein synthesis in which "exchange" plays a role. That is, according to this idea, synthesis involves (in part) reversible reactions. These are what we observe under those experimental conditions typical of "exchange." Under other conditions, additional steps are superimposed and we get net synthesis. Thus exchange and synthesis are inter-linked as parts of the same process. I wonder if Dr. Gale would care to comment.

DR. GALE: No, sir. I don't think I would.

DR. GUNSALUS: With the advent of caution in the induced enzyme-cell free protein synthesis field, one generalization would perhaps be safe and seem warranted: that is, the summary should contain a few well-chosen words of caution that the number of enzymes studied and of the systems visualized are not sufficient to draw conclusions. This replaces the required introductory reference to the ferrous-ferric system in all presentations regarding oxidation-reduction studies.

## Part II

*METABOLISM OF GLUTAMIC ACID, PROLINE,  
ORNITHINE, CITRULLINE AND ARGININE*





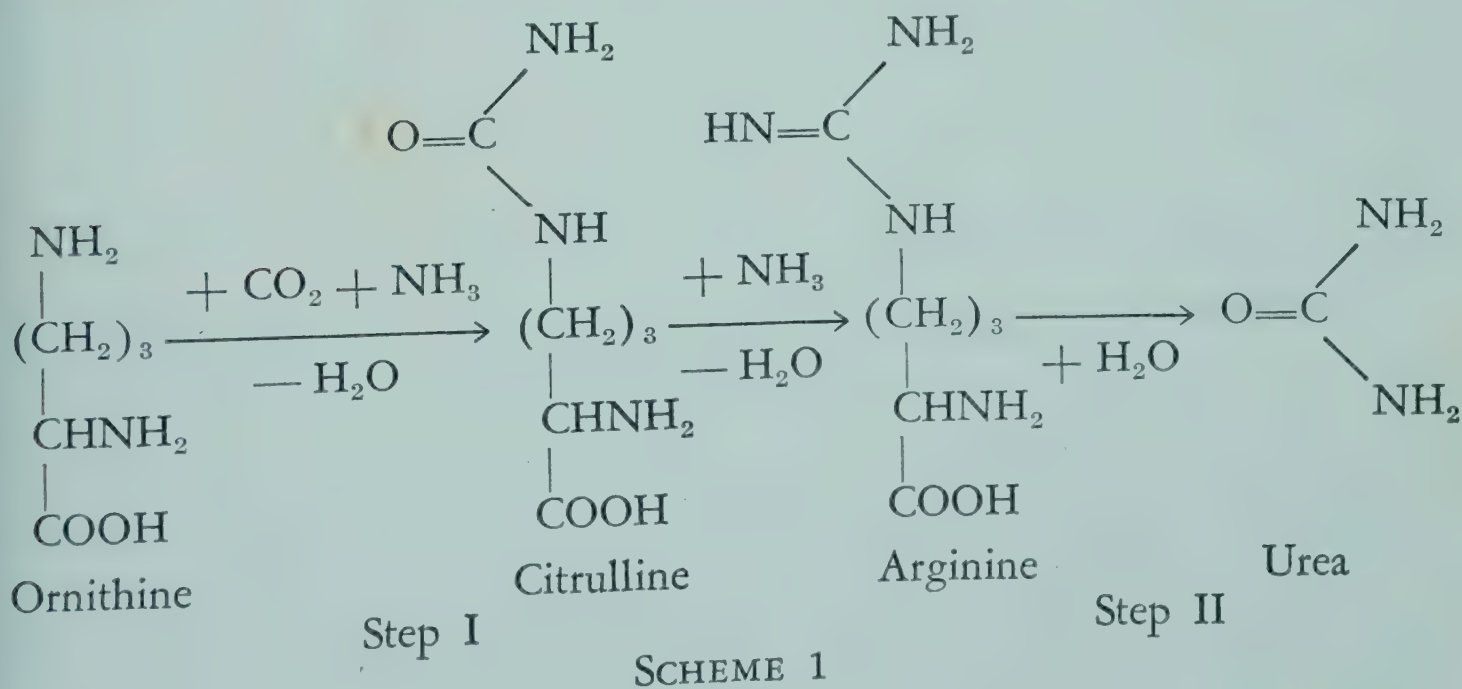
# ARGININE METABOLISM AND INTERRELATIONSHIPS BETWEEN THE CITRIC ACID AND UREA CYCLES \*

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THE AMIDINE GROUP of arginine represents but a small portion of the arginine molecule; it is, however, endowed with high metabolic lability and with a large experimental literature. The metabolism of ornithine will be treated by others during the Symposium, and the present discussion is confined to a consideration of biological functions related only to the synthesis and degradation of the amidine group.

Our recent explorations of arginine metabolism stem from Kreb's formulation of the ornithine cycle (57). He visualized the synthesis of arginine, shown in Scheme 1, as taking place by the successive additions of carbon and nitrogen (in the form of  $\text{CO}_2$  and  $\text{NH}_3$ ) to



SCHEME 1

\* Investigations carried out in the author's laboratory were supported by grants-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.



ornithine, and then to citrulline. He assigned also a primary function to arginine, as the immediate precursor of urea in the mammalian liver, mediated by arginase. The cycle explained, moreover, how a small amount of ornithine facilitates the formation of a large amount of urea through the participation of carrier compounds which are regenerated by the repeated turnovers of a cyclic process (55).

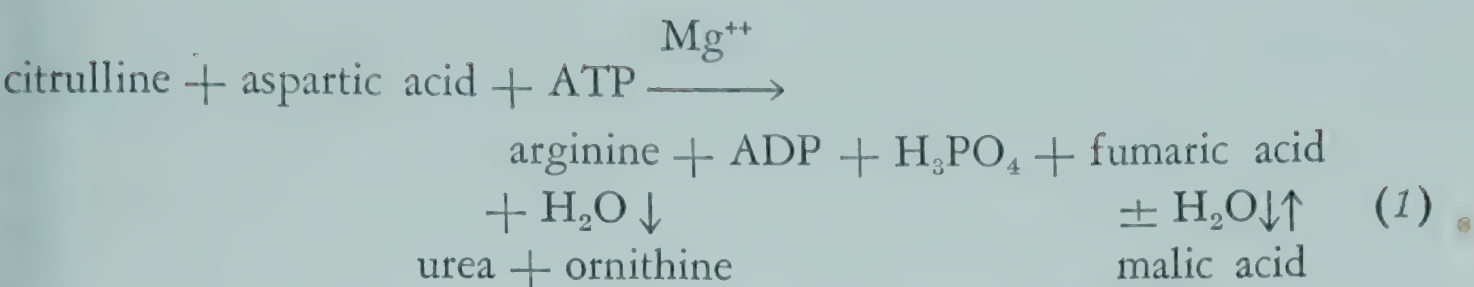
The formation of urea was known to require the expenditure of a large amount of energy, and at this stage of progress questions remained as to how the endergonic requirements are fulfilled, what the nature of the energy coupling might be, and what mechanisms are involved in the attachment of carbon and nitrogen.

In the intervening years, arginine synthesis and urea formation have gone through many stages of investigation; experiments with tissue slices (34, 57) and intact animals (18, 31, 76) were followed by elucidation of the experimental conditions for obtaining active synthesis in respiring homogenates (20-23), and, finally, in cell-free, soluble preparations (38, 68).

With respect to the mammalian systems, my plan is to reverse this sequence and to present first the present status of the detailed reaction mechanisms, as acquired through investigations of isolated systems, and then to consider what their implications may be under the more complex conditions which prevail in respiring tissue preparations and intact cells.

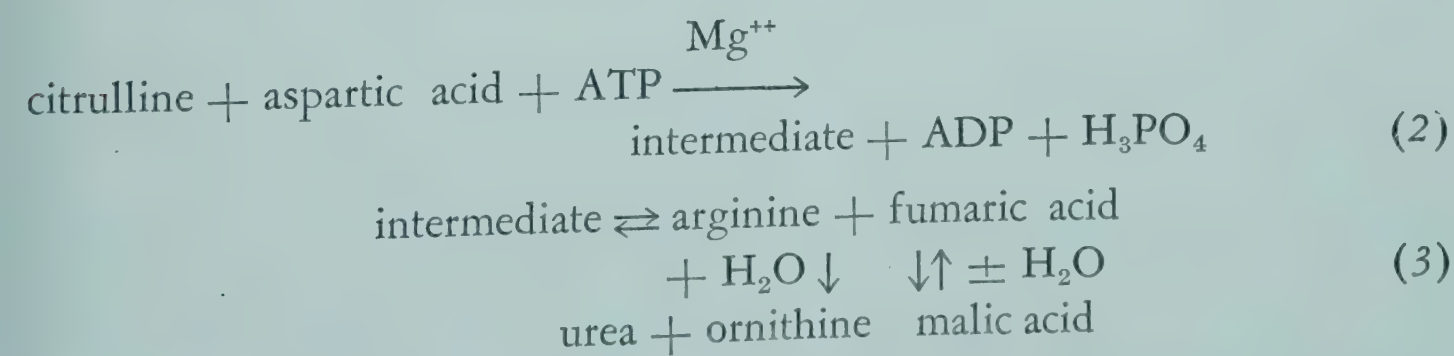
#### ARGININE SYNTHESIS FROM CITRULLINE

When liver tissue is exposed to acetone, the respiration associated with the oxidation of the intermediates of the citric acid cycle is lost, and with it disappear also the capacities for coupled phosphorylation and hydrogen transport to oxygen via the cytochromes. Crude, soluble extracts of acetone-dried liver are still capable of synthesizing arginine from citrulline, and do so anaerobically. In addition to the arginine-synthesizing enzymes, such preparations also contain arginase and fumarase, and can carry out the following over-all reaction, corresponding to Step II of the ornithine cycle:



The nitrogen atom acquired by citrulline can be donated only by aspartic acid, and not by  $\text{NH}_3$  or glutamic acid, as the earlier slice and homogenate experiments appeared to indicate, nor by any other amino acid tried. It turned out also that the synthesis of arginine from citrulline is an endergonic reaction, the energy for which is specifically derived from phosphate-bond energy, for the reaction requires  $\text{Mg}^{++}$  and utilizes at least one ATP per mole of arginine. In respiring tissue, the ATP was adventitiously generated by coupled phosphorylation, and the synthesis could not, therefore, proceed anaerobically. Part of the energy required for urea formation is related to the conversion carried out by reaction (1).

*Stepwise Conversion of Citrulline to Arginine.* As might be suspected from the fact that two products are formed from two amino acid substrates, reaction (1) proceeds through the formation of an intermediate. Two separable enzymatic steps are involved, and after fractionation of crude extracts, one fraction is obtained which catalyzes the accumulation of the intermediate in the presence of ATP. The appearance of inorganic phosphate accompanies the disappearance of citrulline and aspartic acid. On incubation with a second fraction, the intermediate is converted to arginine and fumaric acid (64, 70). The two steps thus consist of a condensation, reaction (2), followed by a cleavage, reaction (3).

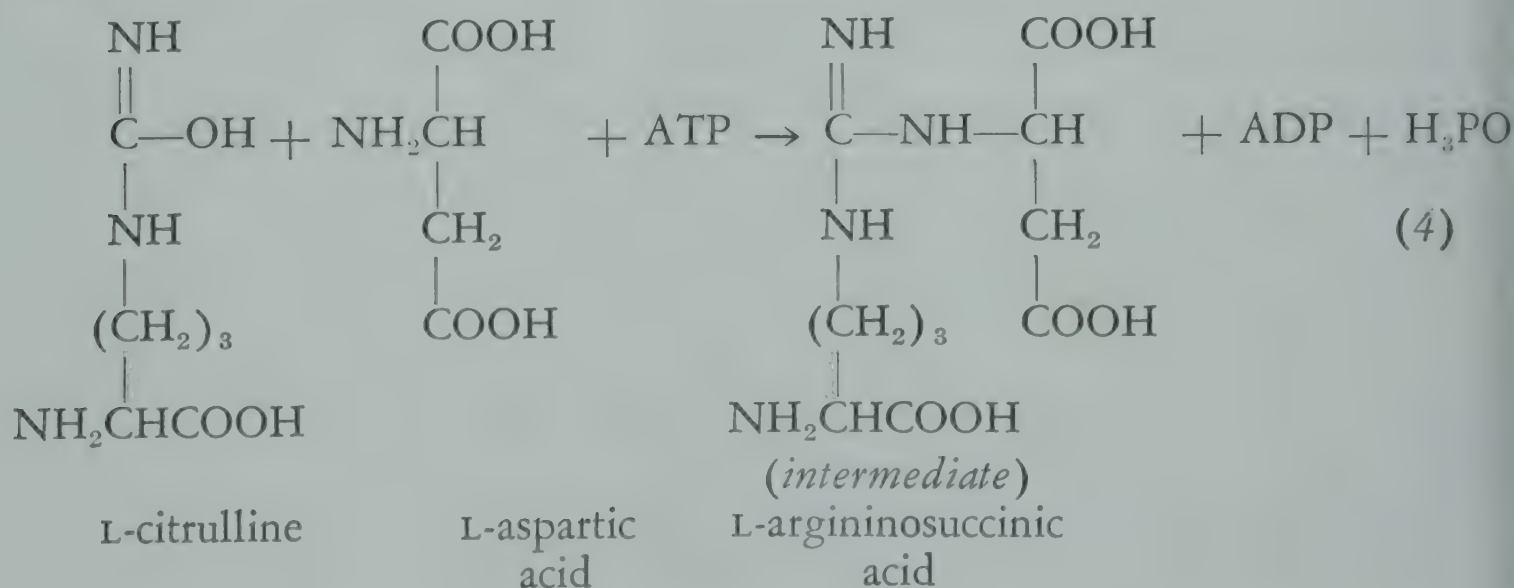


*Mechanism of Nitrogen Transfer.* The mechanism of transferring



nitrogen from aspartic acid to citrulline differs from amino acid-keto acid transamination in almost every feature. In the pyridoxal-dependent mechanism, what is, in essence, an oxidative deamination, is balanced by a reductive amination; the transfer is completely reversible, it is carried out in a single enzymatic step, and there is only a small net change in free energy. Citrulline, on the other hand, acquires nitrogen by forming a stable C—N bond through a reaction which is endergonic, involves a phosphate transfer, and is ostensibly irreversible. This is followed by a reaction of another type, which removes the carbon chain of aspartic acid without changing the oxidation level, and which is only weakly endergonic.

*Mechanism of Condensation.* The intermediate, argininosuccinic acid, is a guanidine derivative, and the reaction by which it is formed is therefore shown as a condensation between aspartic acid and the tautomeric, isourea form of citrulline, by analogy with the chemical synthesis of guanidines from amines and S-methyl or O-methyl isourea.



Here, too, there is little similarity with other ATP-dependent condensations involving carbon and nitrogen, as they occur, for example, in the synthesis of glutamine (26-28, 83, 84) and glutathione (82, 94), where a carboxyl group is transformed to the energy level of an acyl amide. In the arginine-forming mechanism, phosphate-bond energy is utilized in transforming the ureido carbon to the amidine level. The exact nature of the phosphorylating mech-

anism holds considerable interest, for the primary synthesis of the guanidine group is accomplished in the condensation reaction. The step which follows merely converts a disubstituted to a monosubstituted guanidine.

A most attractive hypothesis is one which involves the phosphorylation of citrulline, by ATP, at the ureido carbon, to be followed by a condensation in which the elements of phosphoric acid are split off. A direct phosphorylation of this kind would tend to displace the existing equilibrium mixture of citrulline tautomers in favor of the isourea form. An energy barrier may lie just in this tautomerization.

My colleagues and I undertook a purification of the condensing enzyme system in order to make a further study of the mechanism, and found, during the purification, that two enzyme components are required for the condensation (65, 71). These are almost completely separated after fifty-fold purification of one component. The second one is lost through instability and fractional removal, but can be replaced by a purified fraction obtained from yeast. We hoped that one component would be associated with amino acid phosphorylation, detectable prior to the liberation of inorganic phosphate. Phosphorylation was investigated with each enzyme component and each amino acid in various combinations. ATP was employed as the exclusive high-energy phosphate donor and the remaining ATP was estimated at the end of the incubation by a specific enzymatic method in order to follow any phosphorylation which might otherwise remain undetected (71).

We found that the utilization of ATP and the liberation of inorganic phosphate occur together, but only when condensation also takes place. No evidence was obtained that a phosphorylated amino acid was formed which could exist in the free form. Since the equilibrium position of the phosphorylation may be too unfavorable to allow detection by the methods employed, the possibility should not yet be completely excluded.

The amount of ATP utilized is represented by reaction (4); that is, 1 mole per mole of intermediate formed, but this has not been a



simple matter to establish, owing possibly to contamination by interfering enzymes. The stoichiometry holds, experimentally, in the presence of the purified liver component, where the amount of the second enzyme component is limiting. With larger amounts of the second enzyme present, there is an excess utilization, as measured by the appearance of inorganic phosphate, which varies with the experimental conditions, and tends to approach a value of 2. The excess above 1 is less when ATP is kept at catalytic levels. Whether ATP is kept at high or low concentrations, the inorganic phosphate values are increased in relation to condensation by adding yeast enzyme. Although this seems to indicate that the yeast enzyme affects phosphate transfer in some way, it is too doubtful to be taken as evidence that phosphorylation occurs as a separate step, for the phenomenon fails to occur if one amino acid substrate is omitted (71).

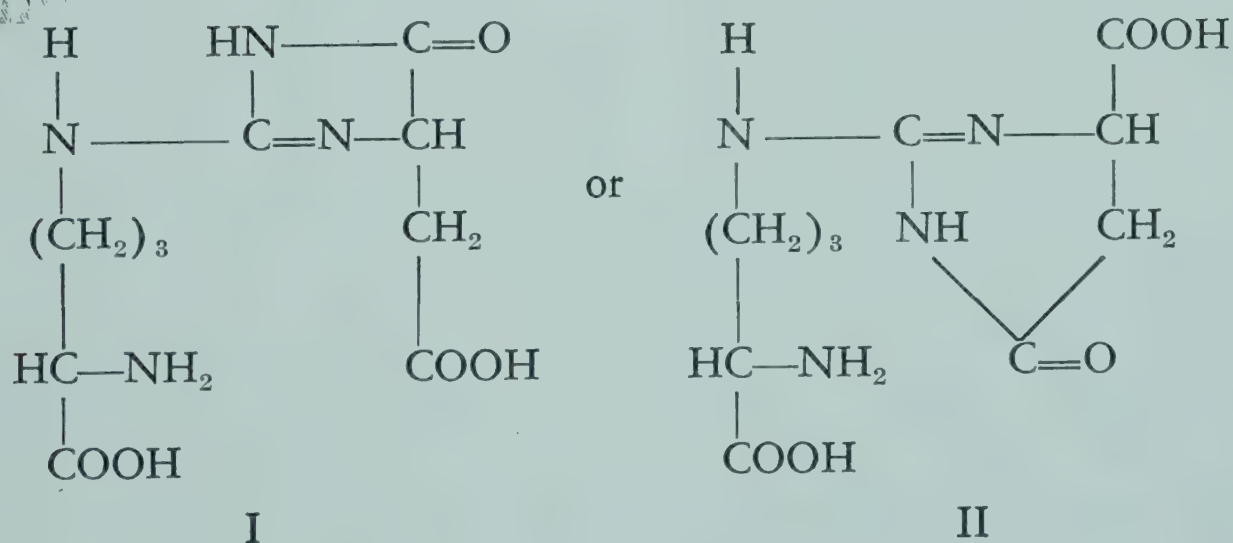
For the present, the phosphorylating aspects of the condensation reaction continue to be illusive. The nature of the interaction with ATP is a point of interest in this connection. The highest rates of condensation are obtained with ATP kept at a catalytic level, maintained by an ATP-generating system. When ATP is present at substrate levels, there is a marked inhibition which appears to be due to AMP, possibly to ADP also, and which is probably competitive (70).

It is becoming increasingly evident that suppression of enzyme activity by high concentrations of ATP is common to many ATP-requiring systems (6, 24, 27, 46, 84, 94). Where the data have been subjected to analysis, in systems free of myokinase, the inhibition has been found to be based on competition with AMP, ADP, or both (27, 82, 84, 94).

*Structure of Argininosuccinic Acid.* The structure of argininosuccinic acid has been substantiated in some detail, and it is significant in the proof of structure that the compound prepared by the condensation reaction is identical with the one prepared from arginine and fumaric acid. The compound is acidic in the isoelectric state, and the dissociating groups have been characterized and

accounted for. The amino acid shows many properties of substituted guanidines. On exposure to hot alkali, for example, there is practically quantitative decomposition to ornithine, aspartic acid,  $\text{CO}_2$  and  $\text{NH}_3$ .

Under certain conditions, argininosuccinic acid rapidly loses its ability to act as substrate for the splitting enzyme. The instability is particularly noticeable in acid solution and at elevated temperatures. The loss of activity is brought about by spontaneous internal condensation with the formation of a cyclic anhydride. After examining the properties of the anhydride, it is still difficult to decide whether the structure is best represented by a five-membered ring (formula I), or by a six-membered pyrimidine ring (formula II). Ring closure can be reversed by weak alkali, the rates in either direction being pH and temperature dependent. The two compounds resemble creatine and creatinine in this respect, and in solution tend to form equilibrium mixtures even at  $0^\circ \text{C}$ . and neutral pH (73).



Absorption spectra of several cyclic guanidino anhydrides are compared in Fig. 1. Guanidinosuccinic acid anhydride, which can, theoretically, form either a five-membered or a six-membered ring, displays an absorption maximum in the short ultraviolet close to those of the five-membered ring compounds, creatinine and guanidinoacetic acid anhydride. The absorption of argininosuccinic acid anhydride is without a maximum in this region, a property that contributes to the uncertainty of ring size (cf. discussion in ref. 73).



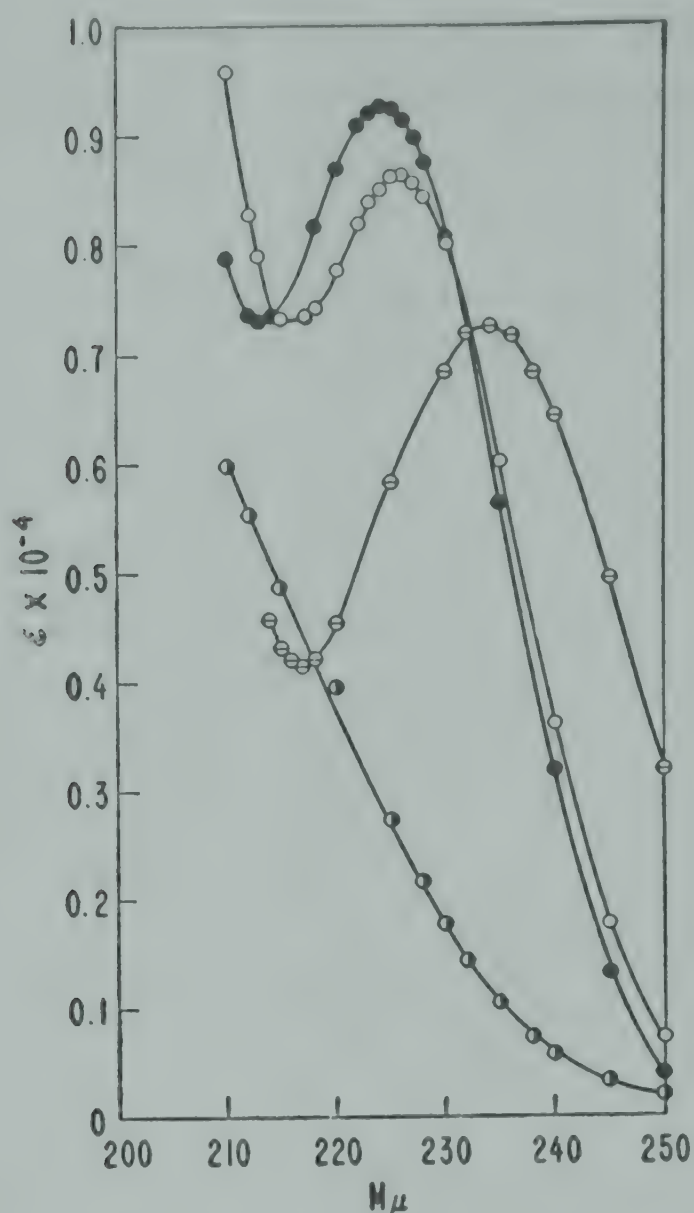
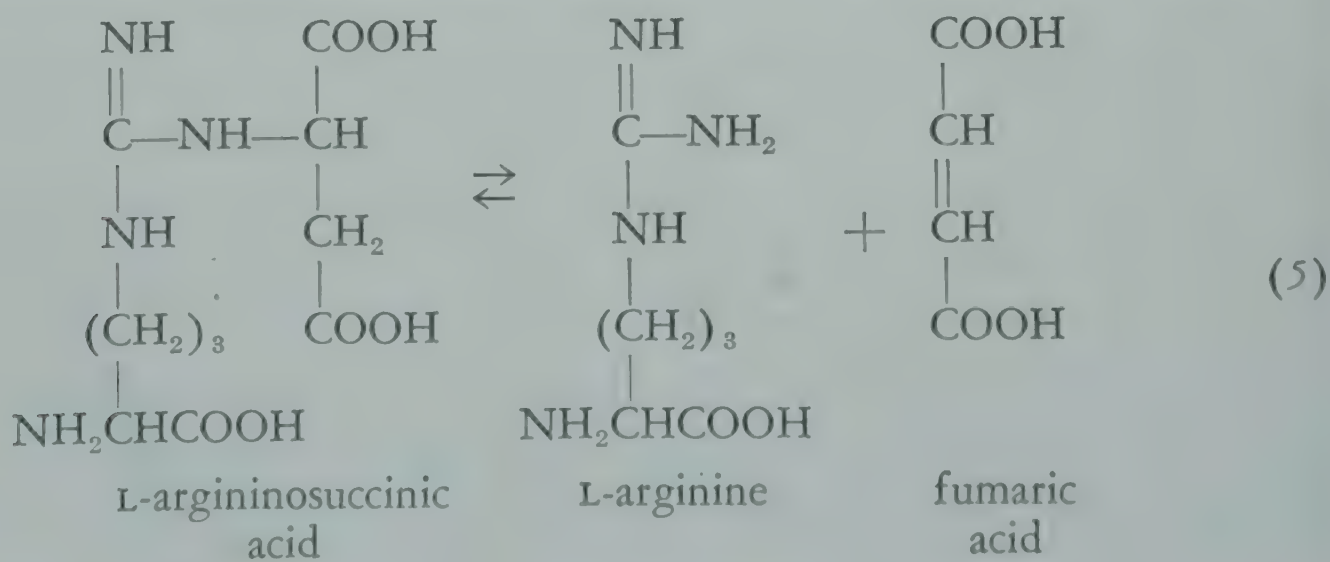


FIG. 1. Absorption spectra of argininosuccinic acid anhydride (●), guanidinoacetic acid anhydride (●), guanidinosuccinic acid anhydride (○), and creatinine (⊖) in 0.01 M potassium phosphate buffer, pH 7.4 (73).

*Cleavage of Argininosuccinic Acid.* This last step is mediated by the splitting enzyme which catalyzes the reversible conversion of argininosuccinic acid to arginine and fumaric acid, reaction (5).



Under suitable conditions, the cleavage can readily be made to proceed to completion by employing an excess of enzyme in combination with arginase to pull the reaction over. This procedure, as a method of quantitative estimation, provides a high degree of specificity. Chemical methods of comparable specificity are lacking.

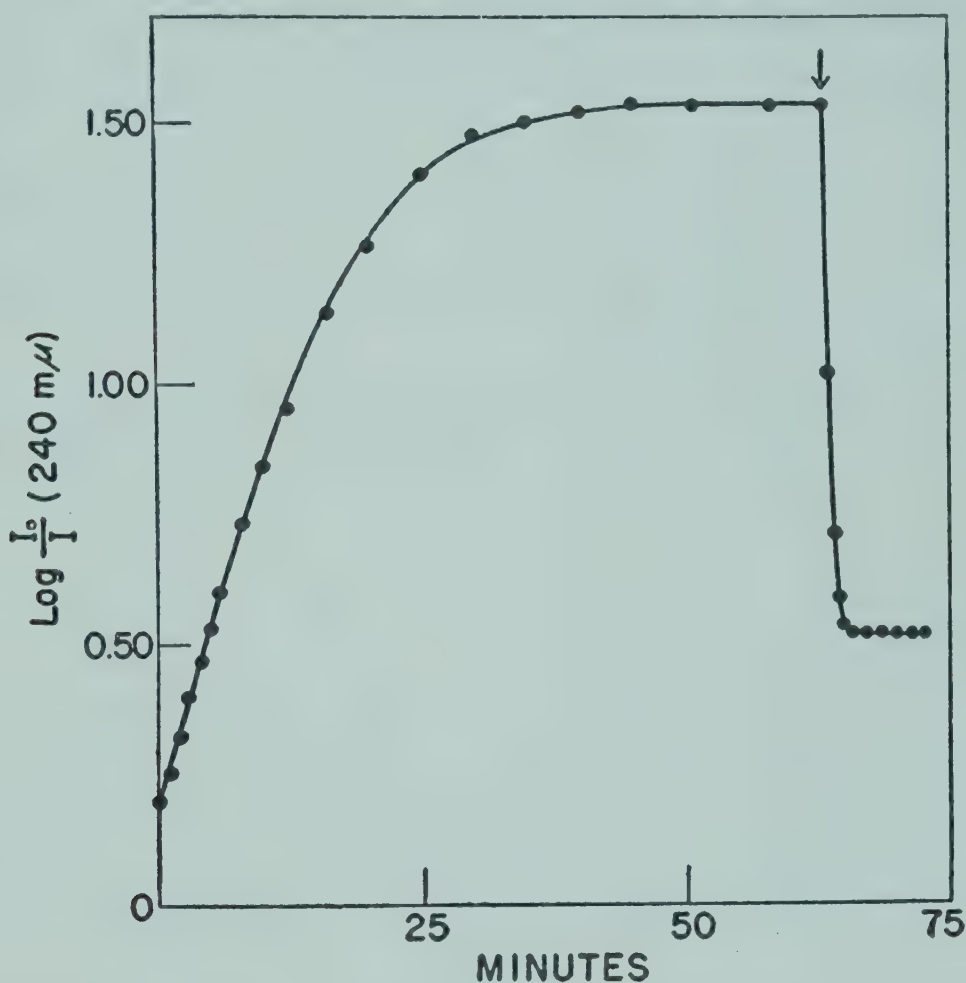


FIG. 2. Spectrophotometric evidence of fumaric acid as the product of enzymatic cleavage of argininosuccinic acid. Purified fumarase was added at the time indicated by the arrow (72).

Fumarase invariably contaminates crude preparations of the splitting enzyme and interferes with the demonstration of fumaric acid as the primary acid product of cleavage, so that the product was originally thought to be malic acid. On removing fumarase, fumaric acid formation can be shown either by permanganate titration, or spectrophotometrically (72). Fumaric acid displays high end absorption in the short ultraviolet. By following the change at  $240 \text{ m}\mu$ , in the presence of splitting enzyme and argininosuccinic acid, there is a rapid increase in optical density, which later approaches a stationary value (Fig. 2). To prove that fumaric acid is indeed the



light-absorbing compound formed, purified fumarase was added at the time indicated by the arrow. The optical density then dropped precipitously to a value, somewhat higher than the initial reading, in agreement with the malate-fumarate equilibrium.

*Properties of the Splitting Enzyme.* Both arginase and fumarase interfere with reversal of the reaction. They can be removed from the liver enzyme, which has been purified over 100-fold to determine the equilibrium position and the reaction kinetics (67). In the direction of cleavage, the value for the equilibrium constant,  $K_{eq.} = \frac{(\text{arginine})(\text{fumarate})}{(\text{argininosuccinate})}$  is  $11.4 \times 10^{-3}$ , corresponding to a  $\Delta F^0$  of +2800 cal., as was to be expected from the ease of reversal. The reaction is seen to be weakly endergonic in the direction of cleavage.

The composition of the equilibrium mixture is particularly sensitive to concentration in this type of first-order reaction represented by  $A \rightleftharpoons B + C$ . In very dilute solutions, about 0.002 M., only one-tenth of the intermediate remains intact at equilibrium, while at higher concentrations, 0.125 M, the proportion of argininosuccinic acid to split products is approximately 3 to 1 (Table 1).

TABLE 1  
EQUILIBRIUM CONSTANT OF SPLITTING REACTION (67)

Initial concentration			Final concentration			$K_{eq.} \times 10^3$
Argininosuccinic acid	Arginine	Fumarate	Argininosuccinic acid	Arginine	Fumarate	
2.5	4.8	4.9	2.5	4.8	4.9	9.4
5.0	4.8	9.7	4.5	5.2	10.1	11.7
5.0	9.6	4.9	4.5	10.0	5.3	11.8
0.0	9.9	9.7	3.2	6.6	6.4	13.2
0.0	9.9	4.9	1.9	7.9	2.9	12.1
2.2	0.0	0.0	0.3	1.9	1.9	12.0
8.6	0.0	0.0	2.9	5.7	5.7	11.2
0.0	125.0	125.0	92.2	29.9	29.9	9.7
Average .....						11.4

All concentrations are expressed as micromoles per ml.

The effect of concentration on the equilibrium composition explains the ease with which large amounts of argininosuccinic acid can be prepared through reversal of the reaction (67, 73). The enzyme activity in pea seeds (25) and *Chlorella* (89, 91), which catalyzes the formation of this compound from arginine and fumaric acid, also catalyzes the cleavage (72, 91), and the enzyme thus appears to be identical with the splitting enzyme of mammalian tissue.

The enzyme-substrate affinity is about ten times as high for argininosuccinic acid as for the products of cleavage, a property which also tends to favor cleavage in dilute solution. If the reaction rates in each direction are compared at substrate saturation, the formation of the intermediate is 1.4 times as fast as the cleavage. These properties may hold future interest, should it be found that argininosuccinic acid participates in the formation of metabolites other than arginine.

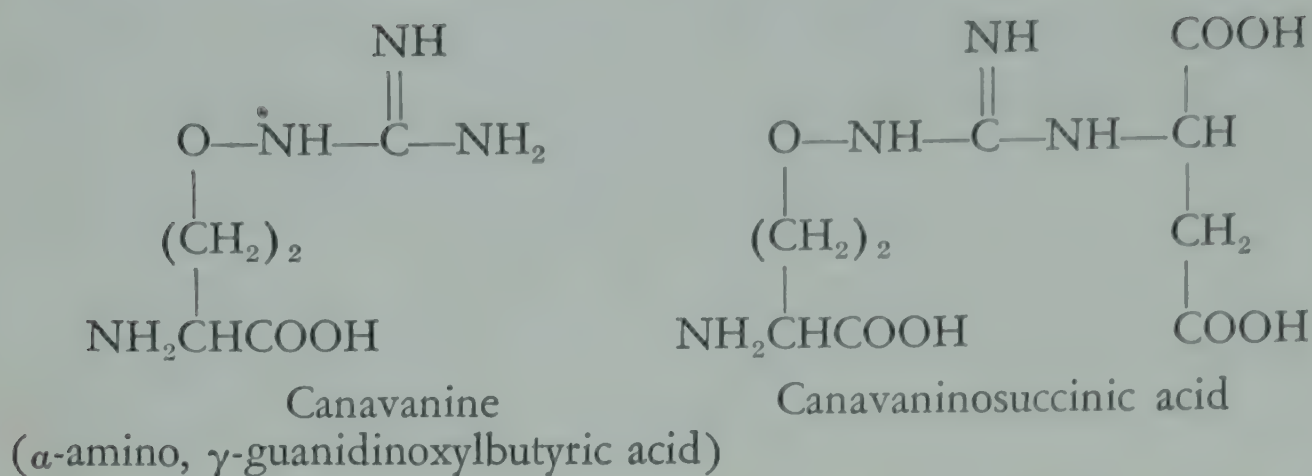
*Comparison with Aspartase Mechanism.* As far as mechanism of action is concerned, there is considerable similarity between the action of the splitting enzyme and the action of aspartase, reaction (6).



They have about the same equilibrium constant, and both catalyze the rupture of a C—N bond with the formation of fumaric acid and a nitrogenous base. Aspartase is not known to occur in mammalian tissues, but it was of interest to see whether highly purified splitting enzyme can utilize aspartic acid as substrate. No activity could be detected even with a large amount of enzyme and a very sensitive method of detection (67).

*Specificity of Splitting Enzyme.* The enzyme specificity appears to be very narrow (67, 91). No other acids have yet been found to substitute for fumaric acid, and only one compound can thus far replace arginine. This is the amino acid canavanine, found in the jack bean, though not in animal tissues. The product formed by interaction with fumaric acid is, presumably, canavaninosuccinic acid (90).





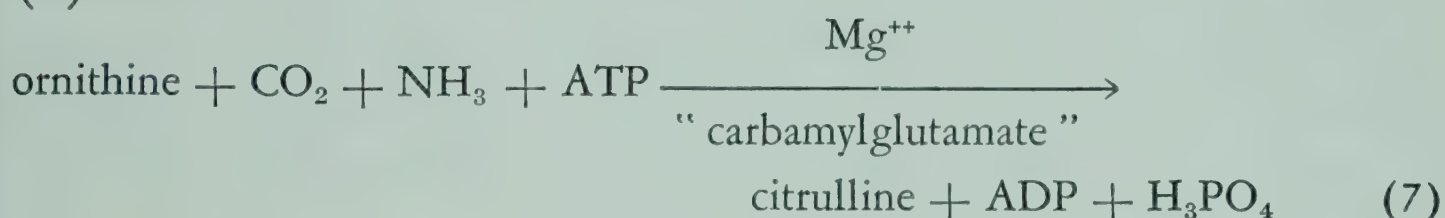
Walker, who used splitting enzyme from *Chlorella* for this purpose, found also that canavanine inhibits splitting enzyme activity, i. e. the formation of argininosuccinic acid; the inhibition can be reversed by arginine, competitively. It had been known that canavanine exerts a strong, arginine-reversible, inhibition on the growth of *Neurospora* (45) and lactic acid bacteria (88). Volcani and Snell, analyzing the phenomenon in bacteria, came to the conclusion that canavanine interfered with the utilization of arginine, whether or not the organisms can synthesize this amino acid (88). At least for the synthesizing strains, it is now possible to localize the site of inhibition at the last enzymatic step, and to explain the basis of the arginine reversal.

#### CITRULLINE SYNTHESIS FROM ORNITHINE

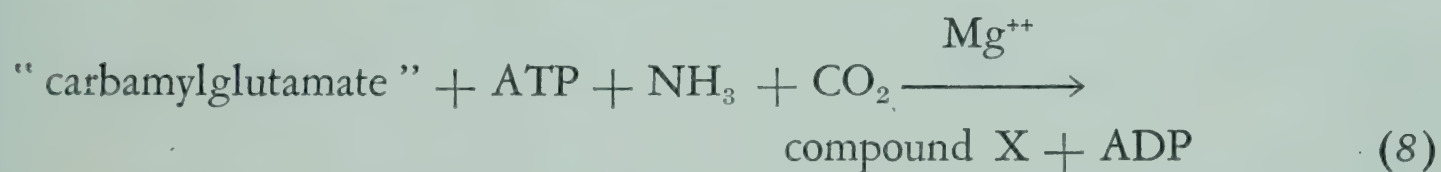
The enzymes which catalyze the conversion of ornithine to citrulline (step I of the ornithine cycle) are associated with the particulate fraction of liver cells, and can be separated from the soluble, arginine-synthesizing enzymes by differential centrifugation of homogenized tissue (23). Their separation by the Wisconsin group permitted, for the first time, an examination of step I in the absence of enzymes which utilize citrulline further. The nitrogen and carbon atoms acquired by ornithine must be supplied in the form of  $\text{NH}_3$  and  $\text{CO}_2$  (36-38), as had been observed with slices. Under the original homogenate conditions, glutamic acid was also required in rather large amounts, but it was soon found that glutamate could be replaced by a small quantity of carbamylglutamic acid (19).

As with the citrulline to arginine conversion, the dependence on

respiration and a particulate enzyme preparation was found to be related to the maintenance of coupled phosphorylation. After destroying the respiratory mechanisms by heat, or by exposure to acetone, Grisolia and Cohen observed citrulline synthesis in liver extracts, under anaerobic conditions, in the presence of  $Mg^{++}$ , ATP, and an ATP-generating system (38, 41). ATP participates as a substrate for an endergonic reaction. As carried out by crude, soluble preparations, the over-all conversion may be expressed as reaction (7).



*Stepwise Conversion of Ornithine to Citrulline.* The participation of carbamylglutamate at the catalytic level suggested that this compound must function as a carrier, and indeed it was found that two participating enzyme systems could be separated by differential heat inactivation and ethanol fractionation (39). The utilization of ATP is associated with the formation of a highly unstable intermediate (35), compound X, reaction (8). The intermediate then reacts with ornithine to form citrulline, and carbamylglutamate is regenerated with the liberation of inorganic phosphate, reaction (9). Since



these reactions will be discussed at length by Grisolia, it is only necessary to note that the energy requirement is confined to the synthesis of compound X, and to the attachment of carbon and nitrogen in a way which seems to allow their transfer to ornithine in the form of a carbamyl group. The catalyst is represented as "carbamylglutamate" for convenience, since several other glutamic acid derivatives can also act as catalysts (40).



## INTERMEDIATES AND ENERGETICS OF THE ORNITHINE CYCLE

When the ornithine cycle was first postulated by Krebs twenty years ago as a three-step mechanism, urea formation could be obtained, experimentally, only with intact liver cells. In the course of subsequent developments, it has become possible to prepare all the participating enzymes in soluble form, separated from each other with some degree of purification and elucidation of reaction mechanism. Five steps now comprise the reaction chain sequence, including reactions (4), (5), (8), (9), and the cleavage of arginine by arginase as the fifth. The number may possibly increase in the future, since there is evidence that the more complex reactions (4) and (8) are each catalyzed by more than one enzyme. In the enlarged cycle (cf. Fig. 3, p. 249), three new compounds appear as intermediates and as catalysts: "carbamylglutamate," compound X, and argininosuccinic acid.

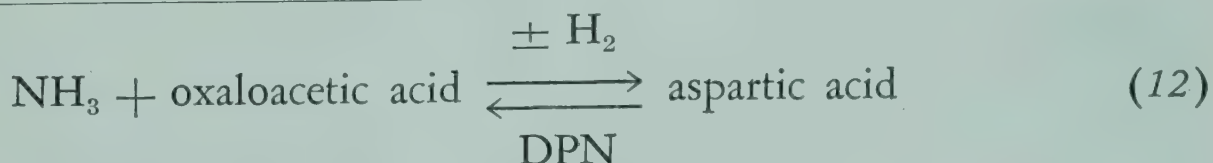
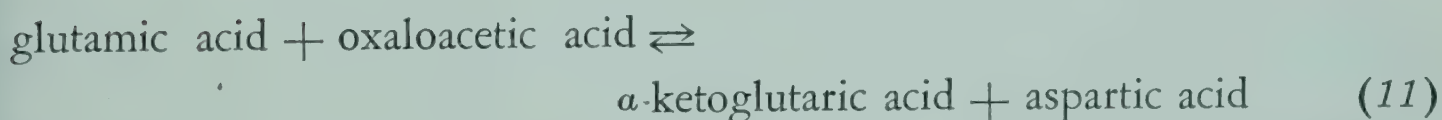
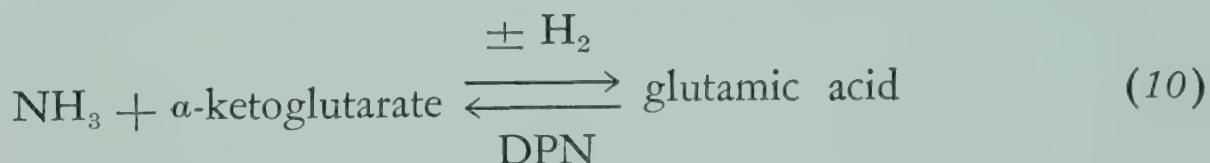
The two high-energy phosphates required represent an energy utilization almost twice as high as the value of 13,800 calories assigned (13) to the free energy gained in the formation of urea from  $\text{CO}_2$  and  $\text{NH}_3$ . The energy requirement can be related to each of the new C—N bonds, the one formed, presumably, in the synthesis of compound X, and the second in the synthesis of argininosuccinic acid.

The energy gained in the synthesis of the carbamyl group must be close to the value assigned to the high-energy bond of ATP, since the degradation of citrulline to ornithine can be coupled to phosphate esterification. Arginine degradation to citrulline proceeds hydrolytically, as far as we know, and we cannot yet evaluate the gain in forming an amidine group from a carbamyl group. Considering that two endergonic steps are involved in converting ornithine to citrulline, the "energy level" of the amidine group is probably very high.

## UREA FORMATION AND THE CITRIC ACID CYCLE

Although study of the isolated systems has brought long-standing problems closer to solution, many new ones have appeared. The transfer of nitrogen from amino acids in general was simple to visualize when it seemed that all nitrogen was incorporated in the form of  $\text{NH}_3$ . The direct oxidative deamination of amino acids was considered to be the only step to precede the ornithine cycle. Now it appears that one-half the nitrogen must be supplied in the form of aspartic acid, and it becomes necessary to reconsider pathways of nitrogen transfer (cf. discussion in 66).

*Nitrogen Transfer from Ammonia.* Direct fixation of  $\text{NH}_3$  into aspartic acid seems unlikely, since mammalian tissues are not known to have enzymes which form aspartate from  $\text{NH}_3$  and fumarate. Fixation can occur by the combination of the following enzymes:



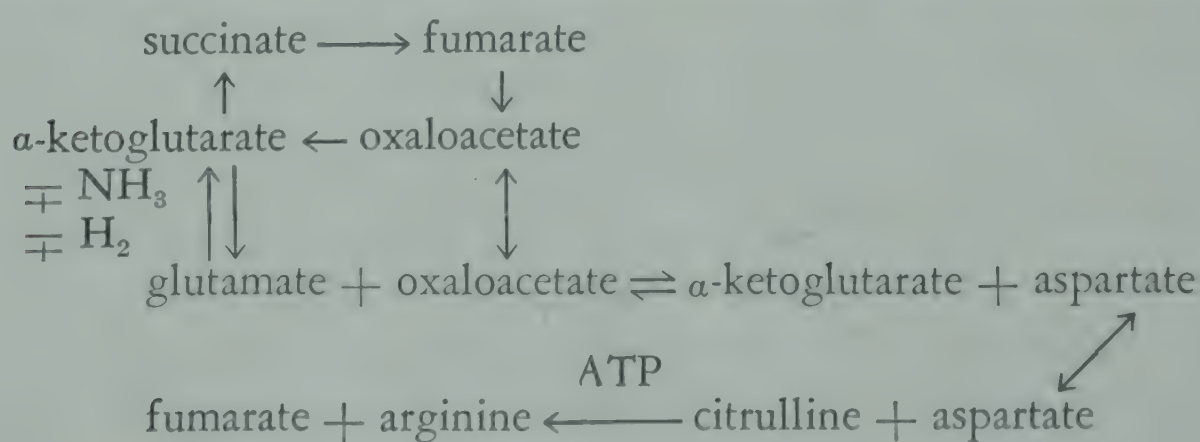
The reductive amination of aspartic acid, represented in the over-all reaction (12), can proceed with the catalytic participation of  $\alpha$ -ketoglutarate and aspartate. Incorporation of  $\text{NH}_3$  into arginine has been carried out by this pathway, anaerobically. All the enzymes required for the entire sequence are present in extracts of acetone-dried liver. They include (a) glutamic dehydrogenase reaction (10), (b) glutamic-aspartic transaminase reaction (11), (c) dismuting reactions for reaction (10), i. e., malic dehydrogenase or triose phosphate dehydrogenase, and (d) the arginine-synthesizing enzymes (69).



*Multiple Function of Glutamic Acid.* In addition to the anaerobic experiments just referred to, examination of respiring preparations also provides evidence which can reflect normal intracellular events. Under the conditions first established by Cohen and Hayano (20) for obtaining rapid urea synthesis in homogenates, glutamic acid was found to be much more effective than aspartic acid. The latter is very poorly oxidized by whole liver homogenates, and to obtain the urea-promoting activity expected of aspartic acid, a respiratory substrate, such as pyruvic acid, must be added to regenerate ATP oxidatively (69).

There are many divergencies among slices, homogenates, and extracts, with respect to the experimental conditions conducive to rapid urea formation. Synthesis is completely dependent on oxygen with the first two, and not with extracts. As the nitrogen donor, slices can utilize  $\text{NH}_3$  and glutamine, but not glutamic or aspartic acids, while homogenates and extracts cannot utilize  $\text{NH}_3$  under the conditions usually employed. Glutamate is not active with extracts and can only replace aspartate in the presence of oxaloacetate and transaminase (68, 69).

The differences just mentioned, along with many others, reflect the presence of active metabolic pathways upon which the operation of the enlarged ornithine cycle depends. Regardless of the form in which the nitrogen is supplied, by having to pass through aspartic acid, urea formation becomes very susceptible to factors which affect either the rate of aspartic acid synthesis or the availability of ATP.



SCHEME 2

When glutamic acid is the nitrogen donor, urea synthesis is high

because the enzymes which catalyze the intervening reactions are in sufficient excess as not to be rate-limiting. Scheme 2 illustrates how a portion of the glutamic acid becomes oxidized, through glutamic dehydrogenase, to  $\alpha$ -ketoglutaric acid, which is then further oxidized by the citric acid cycle to oxaloacetate and converted to aspartic acid by transamination with some remaining glutamic acid (64). Glutamic acid thus furnishes both the nitrogen and the carbon skeleton for aspartic acid and also supplies the ATP through the oxidative steps. It may be seen from the scheme that whenever aspartic acid and a source of ATP are not directly supplied, urea synthesis becomes dependent on the citric acid cycle and on transamination. This explains why inhibitors of respiration also inhibit urea formation.

Other types of inhibition permit a separate analysis of each site of dependence. Malonate, for example, strongly inhibits urea formation when glutamate is the nitrogen donor (11, 20). Since respiration is not greatly reduced, as compared to the decrease in urea, the inhibition must occur by curtailing the supply of oxaloacetate through a block at the oxidation of succinic acid, rather than by appreciable reduction of coupled phosphorylation. Actually, the addition of fumarate, or oxaloacetate, or malate, releases the malonate inhibition, as one would expect (29, 56, 69).

The second site of dependence is revealed by the action of  $\alpha$ -ketoglutaric acid, which exerts its inhibition by displacing the transaminase equilibrium in the direction unfavorable to aspartic acid formation (21, 30, 54, 69). When aspartate is supplied directly (in the presence of pyruvate), the dependence on oxaloacetate formation, and on transamination, is eliminated. Under these circumstances, neither malonate nor  $\alpha$ -ketoglutarate are inhibitory (69).

The scheme also illustrates the course of events which occur with respiring slices, under Krebs' original conditions, where all the nitrogen is offered in the form of  $\text{NH}_3$ , and lactate, which is essential, is also present and acts as a respiratory substrate. The operations of the citric acid cycle will supply  $\alpha$ -ketoglutarate for the primary



fixation of  $\text{NH}_3$  into glutamic acid, and this will be followed by the reactions just outlined for glutamic acid. The fumarate finally formed during arginine synthesis can be returned to the citric acid cycle. Of course, some of the  $\text{NH}_3$  will enter the ornithine cycle, directly, at step I.

The formation of aspartic acid from glutamic acid in respiring tissue has been reported with the further support that the conversion is malonate-sensitive (54, 59).

*Urea Synthesis in Relation to Cell Permeability.* Glutamic and aspartic acids fail to promote rapid urea formation either in slices (57) or in vivo (47). There is general agreement now, among those who have investigated permeability directly, that the two dicarboxylic amino acids penetrate the liver quite slowly (17, 32, 47, 77, 86). A clear relationship, therefore, exists between permeability and utilization. Glutamine and  $\text{NH}_3$ , both of which promote rapid urea synthesis, permeate very readily, as do many other amino acids, and presumably it is in these forms that nitrogen has to be transported to the liver (cf. discussion in 65).

*Physiological Pathways of Nitrogen Transfer.* Once amino acids enter the cell, glutamic acid can act, under physiological conditions, as a funnel for transferring nitrogen from the general pool of amino acids into urea, by the metabolic patterns shown to be characteristic of respiring tissue. The role of  $\alpha$ -ketoglutarate as a general nitrogen acceptor has been reviewed by Dr. Meister. The numerous nitrogen-transferring mechanisms, discussed by him, between glutamic acid,  $\text{NH}_3$ , and glutamine, and also between glutamic acid and many other amino acids, are shown in the upper left of Scheme 3. The enzymes that catalyze these interconversions also bring about the oxidative liberation of all the corresponding  $\alpha$ -keto acids, either directly, or indirectly through general transamination coupled with glutamic dehydrogenase, as suggested some years ago by Braunstein et al. (15, 16). Many of the keto acids are oxidized further via the citric acid cycle and can supply oxaloacetate and high-energy phosphate. The key interlocking enzymes in this scheme, transaminase

The diagram illustrates the metabolic pathways of the urea cycle and its integration with the citric acid cycle. On the left, a vertical bar represents the liver, with four levels labeled from top to bottom: Glutamic acid, Amino acids, Glutamine, and  $\text{NH}_3$ . Arrows indicate the flow of these substances into the metabolic pathways. The urea cycle proceeds through the following intermediates: "Carbamyl-glutamate", Ornithine, Citrulline, Arginino-succinate, Arginine, and Urea. Key reactions include:
 

- $\text{CO}_2$  and  $\text{NH}_3$  reacting with ATP to form "Carbamyl-glutamate".
- "Carbamyl-glutamate" reacting with Ornithine to form Citrulline.
- Citrulline reacting with ATP to form Arginino-succinate.
- Arginino-succinate reacting with Ornithine to form Arginine and Fumarate.
- Arginine reacting with Water ( $\text{H}_2\text{O}$ ) to form Urea and Ornithine.

 The citric acid cycle is shown on the right, with intermediates: Succinate, Fumarate, Malate, Oxalacetate, Citrate, Isocitrate, and  $\alpha$ -Ketoglutarate. Key reactions include:
 

- Oxalacetate and Acetyl-CoA combining to form Citrate.
- Citrate being converted to Isocitrate.
- Isocitrate being converted to  $\alpha$ -Ketoglutarate, a step that produces ATP (indicated by a wavy arrow labeled ~ATP~).
- $\alpha$ -Ketoglutarate being converted to Succinate.
- Succinate being converted to Fumarate.
- Fumarate being converted to Malate.
- Malate being converted to Oxalacetate.

 Connections between the two cycles include:
 

- $\alpha$ -Ketoglutarate from the citric acid cycle entering the urea cycle to form Glutamate.
- Glutamate being converted to  $\alpha$ -Ketoglutarate, a reaction that releases  $\text{NH}_3$ .
- Oxalacetate from the citric acid cycle entering the urea cycle to form "Carbamyl-glutamate".
- Fumarate from the citric acid cycle entering the urea cycle to form Arginino-succinate.

# ARGININE SYNTHESIS IN KIDNEY

The mammalian kidney contains little arginase and appears to be unable to convert ornithine to citrulline. It was discovered by Borsook and Dubnoff that this organ can convert citrulline to arginine (11). The homogenate studies of Cohen and Hayano (20) drew attention to the view that arginine might be formed in both kidney and liver by the same mechanism. Investigations of kidney enzymes, undertaken to examine this question in terms of the mechanism described above, indicate an identical pattern.



Arginine-synthesizing activity, as represented by reactions (4) and (5), is present in the soluble fraction of homogenates, and in extracts of acetone-dried tissue. The condensing and splitting enzymes resemble those of liver and can be separated by the same fractionation procedures (72).

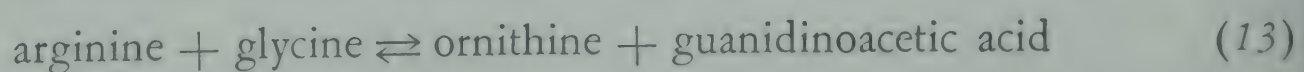
Glutamic-aspartic transaminase, glutaminase, and glutamic dehydrogenase are also very active in kidney tissue. With the exception that the penetration of glutamic and aspartic acids does not appear to be rate-limiting, and the other steps of the ornithine cycle are absent, most of the characteristics of respiring tissue, previously mentioned, also apply to arginine synthesis in the kidney (58, 72).

It is now generally assumed that citrulline is transported to the kidney by the blood stream. Archibald, with his improved methods for the estimation of citrulline and urea, has detected a significant concentration of citrulline in the plasma (4, 5).

#### ARGININE SYNTHESIS IN RELATION TO GUANIDINOACETIC ACID AND CREATINE

The role of arginine in the formation of guanidinoacetic acid and creatine was made clear by Bloch and Schoenheimer (8), and by Borsook and Dubnoff (10, 12), some time ago. The latter group showed that guanidinoacetic acid is formed in the kidney and, subsequently, is converted to creatine by a methylation which takes place in the liver.

Arginine and glycine form guanidinoacetic acid by a reaction called transamidination, reaction (13). It would seem that arginine



represents the primary source of the amidine group, and is available to an acceptor molecule when attached to ornithine.

The synthesis, in crude extracts, showed no requirements other than the amino acid substrates, a behavior which suggested that the amidine group might be transferred with little change in free energy. This has been borne out by the results of Fuld, who recently purified the enzyme and obtained evidence that the reaction is



reversible (33). We have been interested in this problem in New York, during the past year, and have also found reversibility with enzyme purified about fifty-fold (74).

### GENERAL ASPECTS OF ARGININE SYNTHESIS

The important implication of the early isotope experiments carried out by Schoenheimer and his group regarding the origin of the amino acids incorporated into protein and the rapid turnover of amidine nitrogen in accord with the operation of an ornithine cycle, is well known (76).

Experiments with intact animals also provide an interesting correlation with the presence of large amounts of arginase in liver and its absence from other organs. Bloch fed arginine, labeled in the amidine group with  $N^{15}$ , and then isolated arginine from the proteins of different tissues. The lowest concentration was found in the liver arginine (7).

Evidence of this type, obtained from arginine trapped in tissue protein, demonstrates that arginine-synthesizing mechanisms are being drawn upon to supply the needs of protein synthesis in addition to urea formation.

The studies of Srb and Horowitz (85) with mutant *Neurospora* strains, describing an ornithine, citrulline, arginine sequence in this organism, emphasized the presence of an ornithine cycle, since *Neurospora* also contains arginase (and urease). Evidence has accumulated that arginine is synthesized by this pathway in *Penicillium* (9), lactic acid bacteria (88), *Escherichia coli* (1), *Tetrahymena geleii* (43, 93), and other organisms, some of which lack arginase. Wherever it has been investigated, citrulline invariably appears to lie in the pathway of arginine synthesis from ornithine. The reaction chain is then not restricted to urea formation but represents a general pathway, widely distributed in nature, for providing the arginine of cellular protein. The incorporation of this pathway into a metabolic cycle, for the purpose of forming urea, perhaps represents a diversion of general synthetic mechanisms toward one of the special functions of the mammalian liver.



Enzyme distribution is also beginning to provide more detailed evidence. The citrulline-synthesizing enzymes have as yet only been investigated in the livers of urea-forming vertebrates (40). Arginine synthesis from citrulline has been explored more widely. The condensing and splitting enzymes are found in a variety of organisms (Table 2). A number of arginine-requiring bacteria, not included

TABLE 2  
ARGININE SYNTHESIS FROM CITRULLINE  
Enzyme Distribution

Condensing enzymes	Splitting enzyme	References
Mammalian liver	Mammalian liver	70
" kidney	" kidney	72
Yeast	Yeast	60
<i>Neurospora</i>	<i>Neurospora</i>	14
	<i>E. coli</i>	74, 90
	Pea seed	25
	Jack bean	91
	<i>Chlorella</i>	91

in the table, were investigated for splitting enzyme activity by Walker. Activity was absent from strains whose arginine requirement could only be satisfied by arginine, and found to be present in strains whose requirements could be satisfied by citrulline (90).

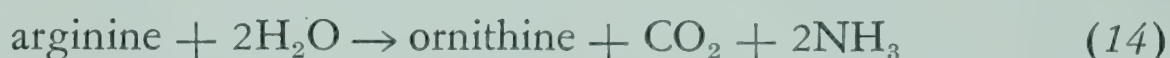
It is interesting that both enzymes are present in *Neurospora*, since the presence of only one mutant, with a block between citrulline and arginine, has been reported (85). Boylen and Fincham have investigated arginine-synthesizing activity in extracts of both the mutant and the wild type. The block appears to be associated with the condensing enzyme. Extracts of the mutant, in contrast to the wild type, show extremely weak ability to form arginine from citrulline, while the splitting enzyme is equally active in both strains (14).

#### DEGRADATION OF ARGININE AND CITRULLINE TO ORNITHINE

The hydrolytic cleavage of arginine to ornithine and urea, by the action of arginase, is the most thoroughly investigated of the enzymatic mechanisms concerned with arginine degradation. There

is no need to discuss the subject here, since there has been no change in the past few years in our concepts of the reaction mechanism or of the primary function of arginase in urea formation.

There have been scattered observations in the older literature that arginine can be degraded enzymatically by mechanisms other than arginase. Citrulline had been isolated from bacterial culture media incubated with arginine (2, 44, 87), and the name arginine desiminase was suggested by Horn (44) for the responsible enzyme. Later, in 1940, Hills observed that washed suspensions of streptococci and staphylococci degrade arginine but not citrulline or most other amino acids, and the streptococci lacked urease (42). The latter observation, and the fact that the ornithine was formed for each molecule of arginine utilized, led Hills to the proposal that the reaction was catalyzed by a single enzyme, distinct from arginase (reaction 14).



The rate of degradation can be extraordinarily high with  $Q_{\text{NH}_3}$  values ( $\mu\text{l. per mg. dry wt. per hr.}$ ) ranging from 50 to 200.

A report by Woods and Trim (92), who investigated *Clostridium welchii*, raised doubt as to the simplicity of the reaction, and later Sekine (3, 78) proposed that the degradation must follow a stepwise sequence, since he had observed both citrulline accumulation from arginine, and citrulline degradation, employing another strain of *Streptococcus faecalis*.

Most strains of *S. faecalis* are impermeable to citrulline. By pre-treatment with acetone or detergent, or by studying other species, several groups of investigators, in England and here, have recently demonstrated that two separable enzymatic steps are involved (48, 61, 75, 81). Citrulline is an intermediate in the degradation, as in the synthesis, of arginine.

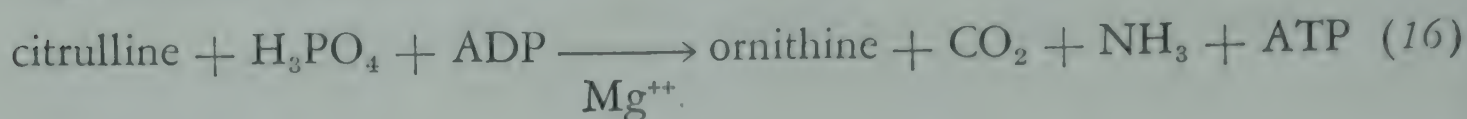
Both steps will shortly be discussed in detail. It is only necessary to mention now that the degradation of arginine to citrulline and  $\text{NH}_3$  (reaction 15) is hydrolytic and appears not to be at all related



in mechanism to the synthesis of arginine from citrulline and aspartic acid (48, 61, 62, 75, 79).

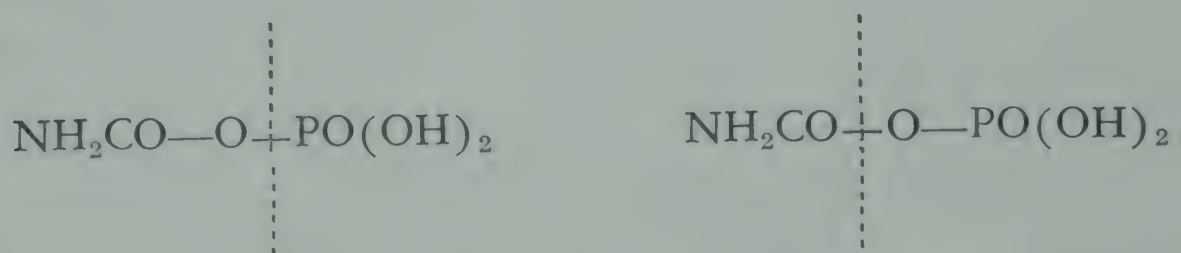


Much greater similarity to mammalian mechanisms is shown in the degradation of citrulline to ornithine,  $\text{CO}_2$ , and  $\text{NH}_3$  (reaction 16).



This step is coupled to the generation of high-energy phosphate (49, 50, 51, 52, 53, 63, 80) and, at least in crude extracts, appears to be reversible (53). In effect, the reaction is a phosphorolysis (52, 53) with ADP as the acceptor, and it undergoes arsenolysis in the absence of acceptor.

The suggestion has been made that the breakdown may proceed through phosphorylated citrulline (51, 63). It is difficult to understand why citrulline phosphate should break down more readily than citrulline or how phosphorylation can take place with inorganic phosphate. Phosphorylation at either of the positions suggested (ureido carbon or terminal ureido nitrogen) would be expected to require high-energy phosphate. It is tempting to visualize the reaction as occurring through phosphorolytic cleavage of the carbamyl group, so as to form an unstable ester of carbamic acid which may not even exist in the free form. A structure of this type is suggested because it could participate both in the degradation and the synthesis of citrulline.



Theoretically, enzymatic cleavage on one side of the ester oxygen would yield high-energy phosphate (anhydro phosphate accepted by ADP), and  $\text{CO}_2$  and  $\text{NH}_3$  from liberated carbamic acid, too unstable to exist as such. Cleavage on the other side would result in a carbamyl group (accepted by ornithine) and inorganic phosphate.

Whether mammalian tissues possess the degradative enzymes is only now coming under investigation; the distribution among microorganisms is very wide. The breakdown of arginine, through citrulline phosphorylase, can provide anaerobic energy sources for bacterial

growth in addition to or in place of glycolysis. Coupled phosphorylation takes place at the substrate level without the primary dehydrogenation associated with the substrate-level coupling of triose phosphate or  $\alpha$ -ketoglutarate oxidation.

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# RECENT ADVANCES IN CITRULLINE BIOSYNTHESIS

SANTIAGO GRISOLIA \* and RICHARD O. MARSHALL

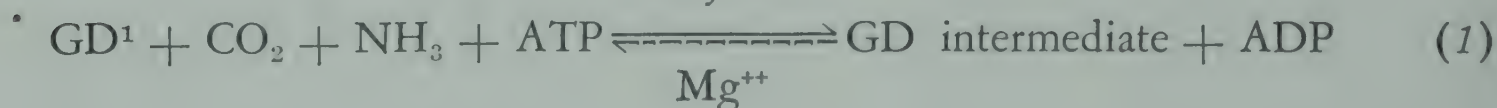
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IN THIS REPORT dealing with the present status of citrulline biosynthesis we shall give special emphasis to the most recent developments of the problem. Our report will be based almost entirely on work carried out in the Department of Physiological Chemistry of the University of Wisconsin from the time of our report here in the summer of 1951 to essentially a few weeks ago.

We brought forward here in 1951 (4) the first evidence for the catalytic mechanism of action of carbamyl glutamate in citrulline biosynthesis. This permitted us to divide the reaction ornithine → citrulline into two main logical steps as indicated in reactions (1) and (2).

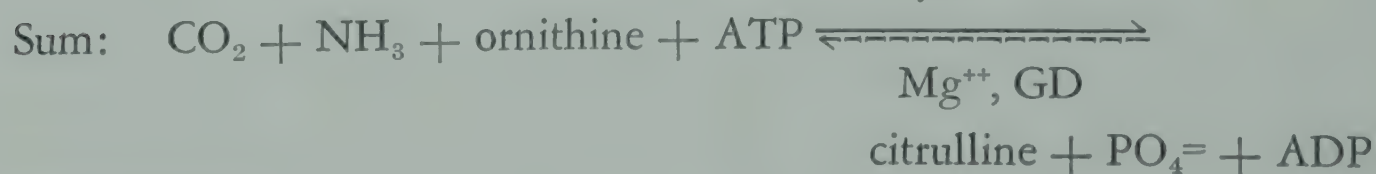
*Enzyme I*



*Enzyme II*



*Enzymes I & II*




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\* Part of the work presented in this paper was done during the tenure by the senior author of an Established Investigatorship of the American Heart Association.

<sup>1</sup> The following abbreviations are used throughout this paper: GD, any of the glutamyl derivatives found to act catalytically in citrulline synthesis (5); ATP, ADP, adenosine tri- and di-phosphate respectively; GD intermediate, any of the intermediates enzymatically formed from GD, ATP, NH<sub>3</sub> and CO<sub>2</sub>; Compound X, GD intermediate formed from carbamyl glutamate (6); CG, carbamyl glutamate; FG, formyl glutamate; CAGD, chloroacetyl glutamate; PG, propionyl glutamate; AG, acetyl glutamate; CHA, cyclohexylamine; PGA, 3-D-phosphoglycerate; M.P., muscle preparation, as described by Ratner and Papas (14); Enzymes I and II, as previously described (6).

Our main concern during this period has been to consolidate and extend the preliminary evidence reported here in 1951 for the mechanism described in reactions (1) and (2).

For the purpose of this presentation we shall concern ourselves with the following points in the order indicated:

- (1) evidence for the biological synthesis of GD;
- (2) evidence for the role of GD in citrulline biosynthesis;
- (3) isolation of GD intermediates;
- (4) nature of GD intermediates;
- (5) enzyme systems catalyzing reactions of GD intermediates.

## 1. EVIDENCE FOR BIOLOGICAL SYNTHESIS OF GD

The first clear-cut evidence for the formation of such an intermediate was shown by us in washed residues of rat liver (7). During a preliminary incubation of the complete system, except for ornithine, required for the synthesis of citrulline, some intermediate accumulates which, when supplemented with ornithine and a soluble liver fraction, permits the reaction ornithine  $\rightarrow$  citrulline to proceed. This does not demonstrate the formation of CG or any other GD compound, but indicates clearly the formation of an active intermediate. More direct evidence for GD synthesis has been obtained as follows:

### A. *Carbamyl glutamate synthesis.*

The synthesis of carbamyl glutamate in the rat by using isotopic dilution techniques both in vivo and in vitro is indicated by the experiments shown in Table 1. In experiments A and A<sub>1</sub> it is seen that radioactive carbon dioxide is incorporated into CG in the presence of properly fortified washed residue preparations. It is also seen that in these experiments the incorporation of C<sup>14</sup>O<sub>2</sub> into citrulline is about 70 times faster than into carbamyl glutamate; this is not surprising, since carbamyl glutamate, a catalytic agent for citrulline synthesis, is most likely synthesized at a slower rate than citrulline. Experiment B shows that the carbon of radioactive formate is also incorporated into carbamyl glutamate. It is possible



TABLE 1

THE INCORPORATION OF  $C^{14}O_2$  INTO CARBAMYL GLUTAMATE AND CITRULLINE,  
AND OF  $HC^{14}OOH$  INTO CARBAMYL GLUTAMATE

Exp.	Compound Isolated	Specific Activity c/m/mM.	Dilution Factor	Corrected Specific Activity c/m/mM.	Micromoles Synthesized
A <sub>1</sub>	Citrulline	2,320	540	$1.16 \times 10^6$	9.0
A <sub>1</sub>	Citrulline	1,160	1,080	$1.16 \times 10^6$	9.0
A	CG	153	17,750 *	$1.16 \times 10^6$ *	.065 *
A	CG	76.4	35,500 *	$1.16 \times 10^6$ *	.065 *
B	CO <sub>2</sub>	$32.0 \times 10^4$		$32.0 \times 10^4$	
B	CG	$30.4 \times 10^3$	685		
B	CG	$7.6 \times 10^3$	2,740		

\* Calculated from the specific activity of citrulline, which is taken to be equal to that of carbamyl-L-glutamic acid.

Experiment A. Washed rat liver residue 6.85 mg. of nitrogen was incubated with the following final concentration of substrate expressed in micromoles: L-potassium glutamate, 200;  $NH_4Cl$ , 40; ATP, 15;  $MgSO_4$ , 50; phosphate buffer pH 7.2, 75;  $NaHC^{14}O_3$ , 60 (1460 c./min./micromole); and potassium ions to bring the medium to isotonicity. Time 20 minutes at 38° C. Conditions for experiment A<sub>1</sub> as for A, except that 30 micromoles of L-ornithine were added. Final volume 8 ml. for A and A<sub>1</sub>. At the end of the incubation 1,175 micromoles of the potassium salt of CG were added, and the CG was reisolated  $2 \times$  after deproteinization. The isolated CG was then diluted by a factor of 2 with non-labeled CG and recrystallized once from water. In experiment A<sub>1</sub> the deproteinized solution after incubation showed a total of 9.0 micromoles of citrulline as measured colorimetrically. An aliquot containing 2.24 micromoles was diluted by a factor of 540 by the addition of 191.6 mg. of nonisotopic L-citrulline, a second aliquot of 2.24 micromoles was diluted by a factor of 1,080 by the addition of 383.2 mg. of nonisotopic L-citrulline. These samples were recrystallized three times before counting. Experiment B was conducted by injecting intraperitoneally 0.5 ml. of a solution containing 1.75 mg. of sodium formate (specific Activity 57.2 microcurie/mg. Na formate) into a 150 g. rat. After 2 hours the animal was sacrificed, and the liver, weighing 8 g., was homogenized in the presence of 2,740 micromoles of potassium CG; CG was then reisolated after deproteinization. After counting, a 100 mg. sample was dissolved and further diluted with 300 mg. of nonisotopic CG, which was then recrystallized and counted. The activity of the respiratory CO<sub>2</sub> during the experiment is reported in the table for purposes of comparison. From the activity of the carbamyl glutamate isolated and of the respiratory CO<sub>2</sub> it can be calculated that there was approximately half a micromole of carbamyl glutamate per gram of tissue. However, this figure should be taken with reservation. (S. Grisolia and P. P. Cohen, unpub.).

to estimate from these data that there was about 0.05-0.5 micromoles of CG per gram of rat liver. However, we may be quite wrong on these figures. It is of interest to remember that carbamyl glutamate acts catalytically in the biosynthesis of citrulline, and if it is not

used appreciably for other reactions, it may be at a higher concentration in mitochondria and possibly even higher at specific sites of this subcellular structure.

### B. *N*-acyl synthesis.

Preliminary experiments to test for the formation of acetyl glutamate in extracts of acetone powders indicate appreciable synthesis, measured by coupling with the citrulline-synthesizing system, as shown in Table 2. We have conducted further work using tissues from a variety of animals to confirm and extend these data. Due to the lack of a specific sufficiently sensitive method for acetyl glutamic acid estimation, we have conducted these preliminary experiments

TABLE 2  
ENZYMATIC SYNTHESIS OF AG IN THE PRESENCE OF THE COUPLING  
ORNITHINE  $\rightarrow$  CITRULLINE SYSTEM

Complete system	Citrulline Synthesis in micromoles	
	Blank	Glutamate
	0.60	2.0
+ KCN	0.64	2.0
+ CoA	0.56	1.75
+ CoA + KCN	0.56	1.8
+ CoA + KCN + glycine	0.56	1.05

10 mg. of protein of a dialyzed water extract of beef liver acetone powder and 3 mg. of M.P. were used per tube. The following substrates expressed in micromoles per 3 ml. were added: ATP, 10; PGA, 40; ornithine, 10;  $\text{NH}_4\text{Cl}$ , 30;  $\text{MgSO}_4$ , 30;  $\text{KHCO}_3$ , 60; potassium phosphate buffer pH 7.3, 30; and, when used, potassium glutamate, 60; glycine, 20; KCN (adjusted to pH 8.0), 40; and CoA, 60 units. Final volume 3 ml. 1 hr. at 38° C. (Grisolia, unpub.).

in all cases by coupling with the citrulline-synthesizing system as a simple assay method. This, however, introduces other difficulties in addition to the difficulty of obtaining quantitative data on the magnitude of acetyl glutamate synthesis, such as interference with the colorimetric assay by some of the components required to demonstrate acetyl glutamate synthesis. In this respect it is worth while to remember that additional difficulties are encountered by competing synthetic reactions, such, for example, as glutamine synthesis. Another indication of a possible competitive reaction is the lower



citrulline synthesis observed in the presence of glycine. These, together with the presence of acylases, make very desirable the purification and separation of the enzyme system from interfering components.

## 2. EVIDENCE FOR THE ROLE OF GD IN CITRULLINE SYNTHESIS

Of a large series of compounds tested (5) (7), a number of N-derivatives of L-glutamic acid will support enzymatic citrulline synthesis from ornithine. The affinity of the soluble enzyme system for the compounds found to be active is in decreasing order as follows: acetyl, chloroacetyl, carbamyl, propionyl, and formyl glutamates (5). All these compounds act catalytically and because of the high affinity of acetyl, ratios of  $\frac{\text{citrulline}}{\text{AG}}$  of the order of 200 can be easily demonstrated (5).

From a consideration of the compounds so far found to be active, it appears that the L-glutamic portion is required specifically and that the amino group substituent must possess a carbonyl group. Further, it appears that the carbonyl group must be capable of enolization. It is not clear, however, why N-formyl glutamate is a less active compound than CG, AG, PG, or CAG.

## 3. ISOLATION OF GD INTERMEDIATES

Because of the spontaneous decomposition of GD intermediates it is necessary to obtain maximum enzymatic synthesis of the intermediates in a short period in order to achieve successful isolation. A study of conditions of incubation has previously been reported (6). The conditions for deproteinization, neutralization of the protein-free supernatant, and calcium salt formation have also been described (6, 8). The method for isolation can be shortened with similar yields of highly pure compound by directly using water extracts of acetone powder, instead of ethanol fractions of the enzyme (each gram of acetone powder is extracted for 20 min. at 0° C. with 5 ml. of water, centrifuged at  $2000 \times g$ , and the precipitate reextracted with 2.5 ml. of water; the combined supernatant

fluids are used as such); also by precipitating the compound through the addition of two volumes of acetone to the supernatant fluid of the calcium-treated deproteinized incubation mixture, instead of fractional precipitation with acetone between 20–50% (which will give ca. 22% purity with a yield of 51%). By precipitating with 2

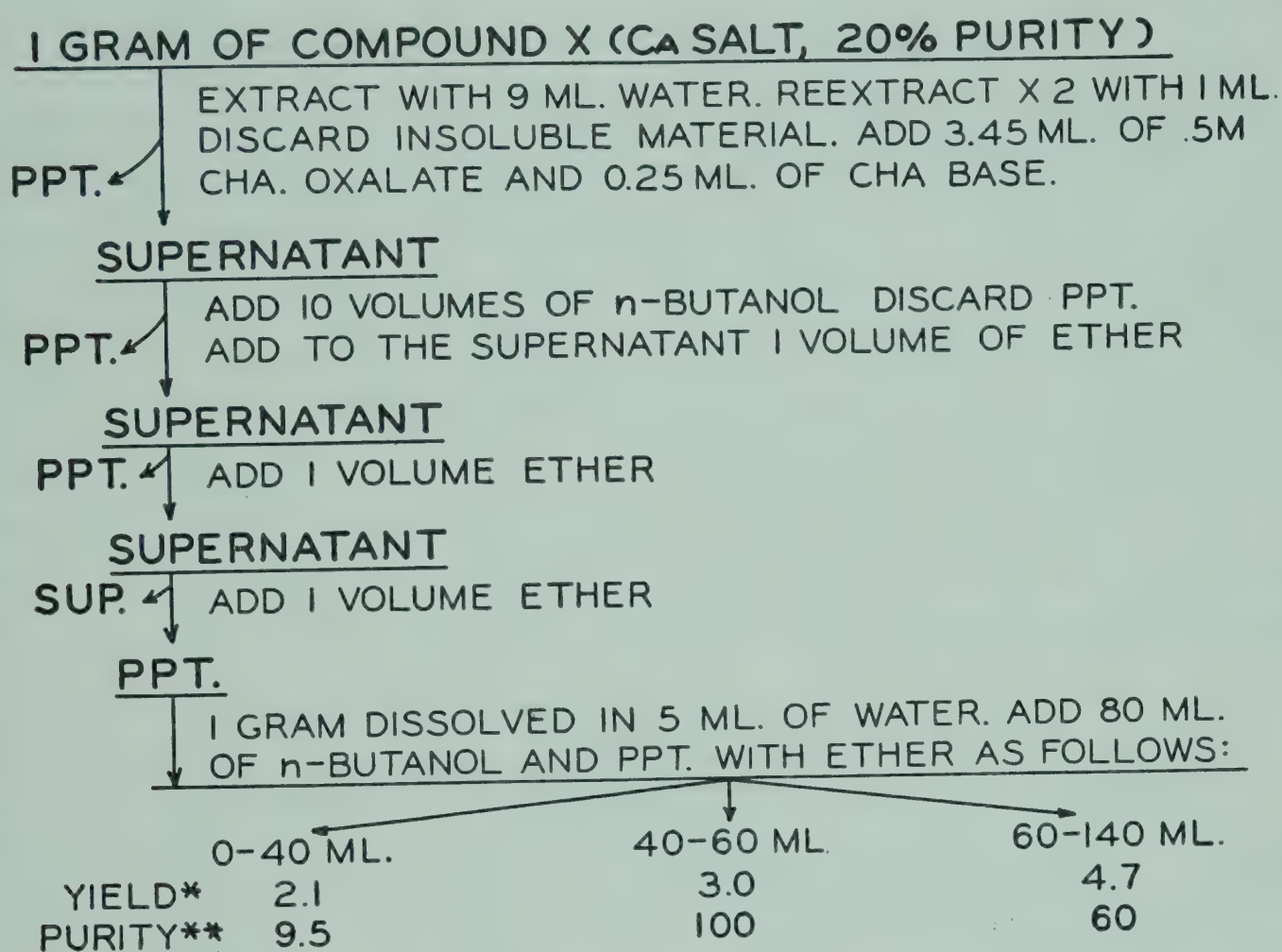


FIG. 1. The isolation of Compound X. (S. Grisolia and R. O. Marshall, unpub.).

\* Yield is calculated from the amount of Compound X present in the incubation mixture prior to isolation.

\*\* Purity is calculated for the tricyclohexylamine salt.

volumes of acetone, compound X of 15% purity is obtained. The 15% pure compound is extracted twice with water (22.2 ml./g. for the first extraction; 11.5 ml./g. for the second extraction). The compound is precipitated by the addition of 1.15 volumes of acetone to the supernatant (yield, 38%; purity, 20%). This method is advantageous for the preparation of sizeable quantities of compound X, since it does not require the preparation of large quantities of ethanol fractions of Enzyme I. From here on the method is essentially as previously outlined (6), and it is further illustrated in Fig. 1. The preparation showing highest purity is obtained in a



yield of 3.0% calculated from the initial compound present in the incubation mixture. Although we have not as yet conducted a similar purification study of GD intermediates other than carbamyl glutamate we would anticipate similar behavior.

#### 4. NATURE OF GD INTERMEDIATES

For practical purposes this discussion will be concerned mostly with the intermediate formed from carbamyl glutamate, called Compound X (6). It is logical to assume that the other GD intermediates will prove to have an essentially similar structure and similar chemical characteristics, as is indeed indicated by the rate of spontaneous decomposition of GD intermediates shown in Table 3. Compound X has been shown to be composed of one mole each of CG, CO<sub>2</sub>, NH<sub>3</sub>, and PO<sub>4</sub><sup>=</sup>, as previously reported (4, 6) and more recently shown with highly purified preparations. The phosphate bond of Compound X appears to be extremely labile.

TABLE 3

RATE OF SPONTANEOUS DECOMPOSITION OF GD DERIVATIVES \*

Time	CGD	$k \times 10^{-2}$	AGD	$k \times 10^{-2}$	FGD	$k \times 10^{-2}$	CAGD	$k \times 10^{-2}$
min.	$\mu M.$		$\mu M.$		$\mu M.$		$\mu M.$	
0	5.25		3.68		3.72		3.58	
30	3.34	1.50	2.24	1.65	2.44	1.39	2.12	1.75
60	2.08	1.54	1.45	1.55	1.44	1.59	1.37	1.60
90			.91	1.56	1.05	1.41	.88	1.56
120	.87	1.50			.73	1.36		
Average $k$ **		1.52		1.59		1.44		1.64

3 ml. aliquots of CGD were incubated at 38° C. at pH 7.2 for the time periods indicated in the table and were enzymatically analyzed as previously described (6).

2 ml. aliquots were used for AGD and CAGD and 2.4 ml. aliquots were used for FGD.

\* One must keep in mind that the decomposition rate is affected by a number of components and conditions. Ca<sup>++</sup> will increase the rate slightly, where Ba<sup>++</sup> is twice as effective as Ca<sup>++</sup>. Cyclohexylamine inhibits the decomposition. However, in the experiments reported here conditions have been kept essentially constant and are as previously described for a similar study of Compound X decomposition (S. Grisolia, unpub. and 6).

$$** k = \frac{1}{t} \ln \frac{a}{a-x}$$

From a study of the decomposition rate of Compound X at a pH near neutrality, the following sequence of decomposition was found (4): first,  $\text{PO}_4^{\equiv}$ , then  $\text{CO}_2$ , while  $\text{NH}_3$  remains in a much more stable bond. These experiments have been repeated with purified Compound X, and a typical experiment carried out at pH 5.0 is shown in Fig. 2.

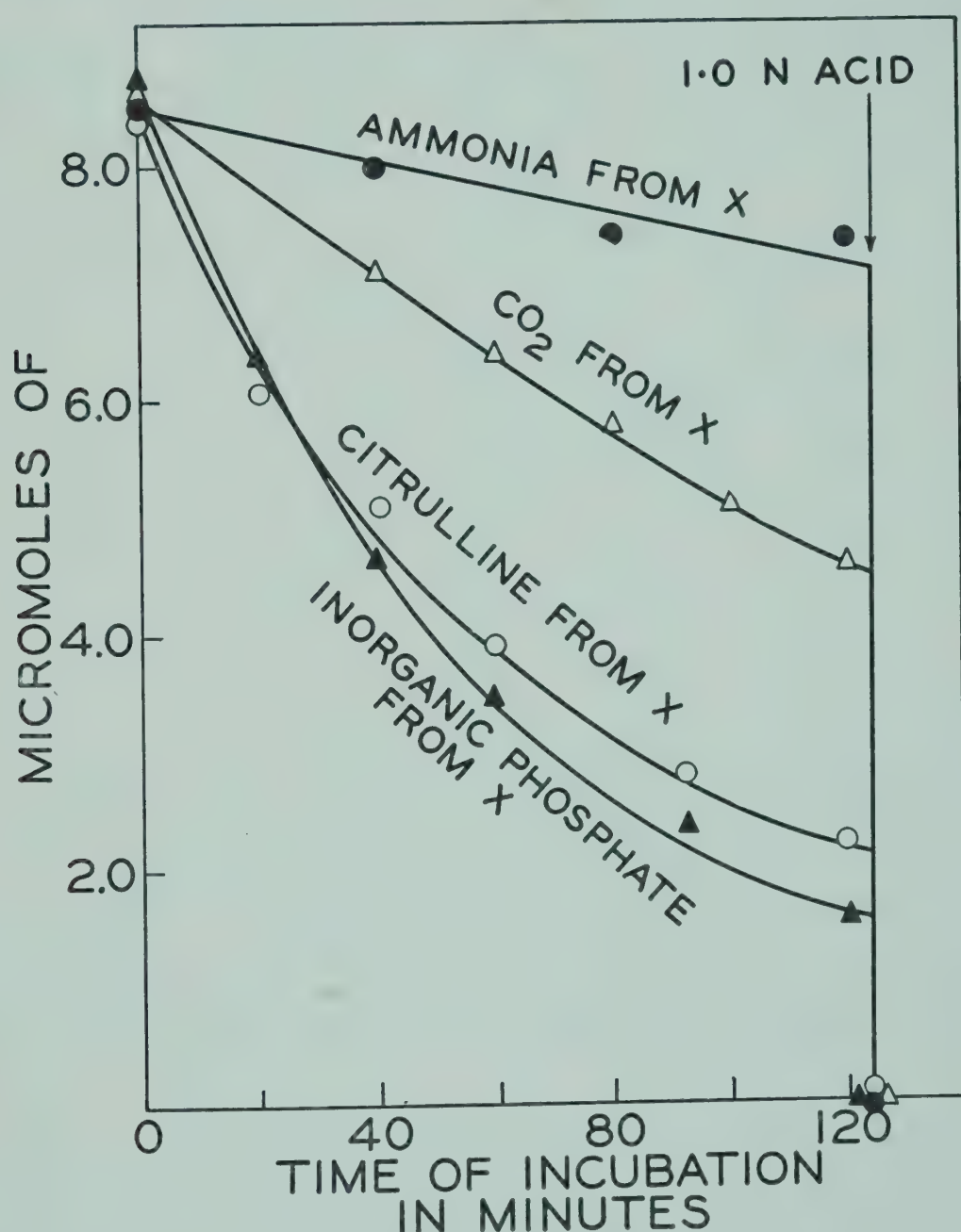


FIG. 2. Spontaneous decomposition of Compound X.

Compound X cyclohexylamine salt of 60% purity was dissolved in water, the pH adjusted to 5.0, and incubated at 38° C. in the presence of .05 M acetate buffer, pH 5.0, for the time periods indicated in the abscissa. Samples were analyzed for Compound X enzymatically as previously described (6). The values obtained are indicated by open circles. Inorganic phosphate was measured by the method of Lowry and Lopez, as previously indicated (4), during the decomposition period, and the values obtained are shown in the figure by the solid triangles. Carbon dioxide liberation was measured manometrically, and the values obtained are shown by open triangles. Ammonia was estimated on eluates from Dowex-2 columns, as previously described and indicated by solid circles (4) (Grisolia, S., Marshall, R. O., and Cohen, P. P., unpub.).



The lability of Compound X has made characterization difficult by limiting the application of such classical methods of identification as degradation and formation of derivatives. The use of carbamyl glutamate selectively labelled with deuterium (9) has, however, allowed us to show that the hydrogen atoms of the carbon chain of the glutamate portion of the molecule are not involved in the reaction leading to the formation of Compound X. To this end, selectively labelled carbamyl glutamate is incubated under the experimental conditions of Table 4, and at the end of the incubation

TABLE 4

STUDY OF THE EXCHANGE OF SELECTIVELY LABELLED CG DURING SYNTHESIS AND DECOMPOSITION OF COMPOUND X.

Exp.	Position of Label	Initial Atom % Excess	Final Atom % Excess	Dilution Factor	Corrected Final Atom % Excess
1	$\alpha$	9.90	.67	14.0	9.40
2	$\beta$	9.45	.69	14.0	9.70
3	$\alpha$ - $\beta$	14.20	.76	19.0	14.40
4	$\gamma$	7.74	.51	15.4	7.85

0.3  $\mu$ M. of the properly labelled CG were incubated for 1 hr. at 38° C. in a volume of 300 ml. with the following components, expressed in mM.: ATP, 0.6; PGA, 5.0; MgSO<sub>4</sub>, 2.5; NaHCO<sub>3</sub>, 4.0; NH<sub>4</sub>Cl, 3.0; Tris buffer pH 7.3, 2.0. In addition 2 g. of enzyme preparation B (6) and 200 mg. of M.P. were added to the incubation mixture. In all cases theoretical conversion (or over 95%) of CG to Compound X was obtained. The incubation mixture was then deproteinized with HClO<sub>4</sub> and allowed to stand over night at room temperature to insure complete decomposition of the intermediate. CG was isolated after dilution, as indicated in the table. (Grisolia, S., et al., 9).

carbamyl glutamate is reisolated after decomposition of Compound X. It must be concluded from the data shown that the carbon-linked hydrogen atoms in carbamyl glutamate are not involved in the reactions leading to the synthesis of Compound X.

Since preliminary evidence indicates that the carboxyl groups of Compound X are free, we have narrowed considerably the possible structure of the compound. It has been shown that Compound X has a reactive carbonyl group, as is evidenced by its reactivity with hydroxylamine and hydrazine. In fact, this reactivity permits the colorimetric estimation of Compound X, since there is linear color



formation upon addition of  $\alpha$ -isonitrosopropiophenone or diacetyl monoxime to samples of Compound X previously treated with hydroxylamine or hydrazine respectively ( $0^{\circ}$  C. for 5 min., followed by heating at  $100^{\circ}$  C. for 5 min.). The color development is conducted essentially as indicated by Archibald (8) for urea or citrulline (1, 2). None of the components of Compound X or any of the cofactors necessary for its synthesis interfere with the colorimetric estimation. The observation of the carbonyl reactivity of Compound X, together with the kinetic decomposition data and the slight inorganic phosphate liberation observed several years ago (7) under conditions leading to synthesis of Compound X, even when some of the necessary components are excluded, led us to reinvestigate this problem. It appeared that it might be possible to obtain some clues as to the order of addition and activation of the components of Compound X by studying the possible increase in inorganic phosphate liberation in a system supplemented with some or all of the components necessary for the synthesis of Compound X in the presence of hydroxylamine. Data on this point are shown in Table 5. It is of interest to keep in mind that at no time has it been possible to show hydroxyamic acid formation under a variety of experimental conditions leading to synthesis of Compound X (4). The table shows little inorganic phosphate liberation when the enzyme system is incubated in the presence of CG, ammonia, and hydroxylamine. However, when  $\text{CO}_2$  is added to CG there is a marked increase in phosphate liberation over the values obtained with CG alone. This increase is very pronounced upon the addition of ammonia, as previously reported (7), and of hydroxylamine; however, in the latter case little synthesis of Compound X occurs.  $\text{NH}_2\text{OH}$  *per se* does not appear to inhibit the formation of Compound X, or the assay system, as indicated by its addition at the beginning or at the end of the incubation under conditions leading to synthesis of Compound X, and also under these conditions it does not effect the liberation of inorganic phosphate.

These experiments suggest that hydroxylamine substitutes for ammonia to form a compound indistinguishable from Compound X but unable to form citrulline. However, if this were the case, there



should be a marked inhibition by hydroxylamine, of a larger order of magnitude than is observed in the table. The second possibility is that hydroxylamine reacts with the incorporated phosphate or  $\text{CO}_2$  as it does with Compound X. For the moment it is not possible to

TABLE 5  
EFFECT OF HYDROXYLAMINE ON INORGANIC PHOSPHATE FORMATION

Additions to System	Compound X formed	Inorganic Phosphate formed
	$\text{m}\mu$	$\text{m}\mu$
CG	0.14	0.0
CG, $\text{NH}_2\text{OH}$	0.16	1.60
CG, $\text{NH}_3$	0.47	0.80
CG, $\text{NH}_3$ , $\text{NH}_2\text{OH}$	0.66	1.60
CG, $\text{CO}_2$	0.28	0.81
CG, $\text{CO}_2$ , $\text{NH}_2\text{OH}$	0.34	8.64
CG, $\text{CO}_2$ , $\text{NH}_3$	2.97	7.96
CG, $\text{CO}_2$ , $\text{NH}_3$ , $\text{NH}_2\text{OH}$	2.42	7.96
CG, $\text{CO}_2$ , $\text{NH}_3$ ( $\text{NH}_2\text{OH}$ added at end of incubation)	3.12	7.50

Each tube contained the following concentrations of components expressed in micro-moles per 2.5 ml: ATP, 12.5;  $\text{MgSO}_4$ , 30; glycyl-glycine buffer pH 7.4, 25; CG, 15;  $\text{NH}_4\text{Cl}$ , 30;  $\text{NaHCO}_3$ , 30;  $\text{NH}_2\text{OH}$ , 100. Each tube contained 10 mg. of protein of preparation B (6). Incubation 25 minutes at  $38^\circ\text{C}$ . Compound X was determined enzymatically as previously described (6), and inorganic phosphate by the method of Gomori (3). Because of the comparative aspects of the experiment no special precautions were taken to insure a  $\text{CO}_2$ -free system in the control experiment. (Marshall, R. O., Grisolia, S., and Cohen, P. P., unpub.).

determine whether the activation of CG to form Compound X proceeds initially by activation via ATP, followed by  $\text{CO}_2$  fixation. It is quite possible that if hydroxylamine reacts with the fixed  $\text{CO}_2$  it might cause a shift of equilibrium of the intermediates formed. The evidence reported here and some additional evidence indicates that we have to concern ourselves only with the  $\text{—N—C—R}$  part



of glutamate derivatives active in citrulline synthesis, and it is possible that the carbamyl oxygen of these compounds might in some way, such as phosphorylation, become reactive or that the fixed  $\text{CO}_2$  might contribute the reactive carbon, since cyclization of the

carbon chain of carbamyl glutamate does not appear probable. However, we are still in the dark regarding the true structure of Compound X. Experiments with  $O^{18}$  in the oxygen of the carbamyl group of CG will be extremely helpful in clarifying the structure of the intermediate, as will be the study of hydroxylamine and hydrazine derivatives of Compound X. It also appears of interest to study the possible formation of hydroxylamine or hydrazine derivatives of the CG-phosphate or CG-phosphate- $CO_2$  compounds. A simple test for this formation suggests itself, namely, the use of the color reactions, already mentioned, with isonitrosopropiophenone or diacetylmonoxine.

#### 5. ENZYME SYSTEMS CATALYZING THE KNOWN REACTIONS OF THE GD INTERMEDIATES

Whether or not the same enzyme system activates CG, CAG, AG, PG, and FG remains to be investigated. However, preliminary evidence indicates that at least one of the enzymes of the system may be common to both carbamyl and formyl glutamate. Because of the lower affinity for formyl glutamate, under the conditions of Fig. 3, it is possible to demonstrate competitive inhibition of citrulline synthesis by formyl glutamate in a system with low levels of carbamyl glutamate.

The soluble enzyme systems required for the biosynthesis of citrulline are present in the liver of all vertebrates so far investigated except birds. No evidence has been obtained for the extrahepatic existence of the enzyme systems catalyzing reactions (1) and (2) in mammalian tissues so far investigated. Recently a great deal of attention has been given to the so-called "ureidase" reaction which in bacteria cleaves citrulline to ornithine,  $CO_2$ , and  $NH_3$  with the production of high-energy phosphate bonds (12) (15). The possible relation of the bacterial enzyme to the mammalian enzymes responsible for reactions (1) and (2) have been hinted at (12). Very recently Eggleston and Krebs (pers. commun.) have shown arsenolytic cleavage of citrulline in mammalian tissues. Professor Krebs indicated to us also that in the presence of ornithine



decarboxylase from *Escherichia coli* preparations a phosphorolytic cleavage of citrulline can be shown.

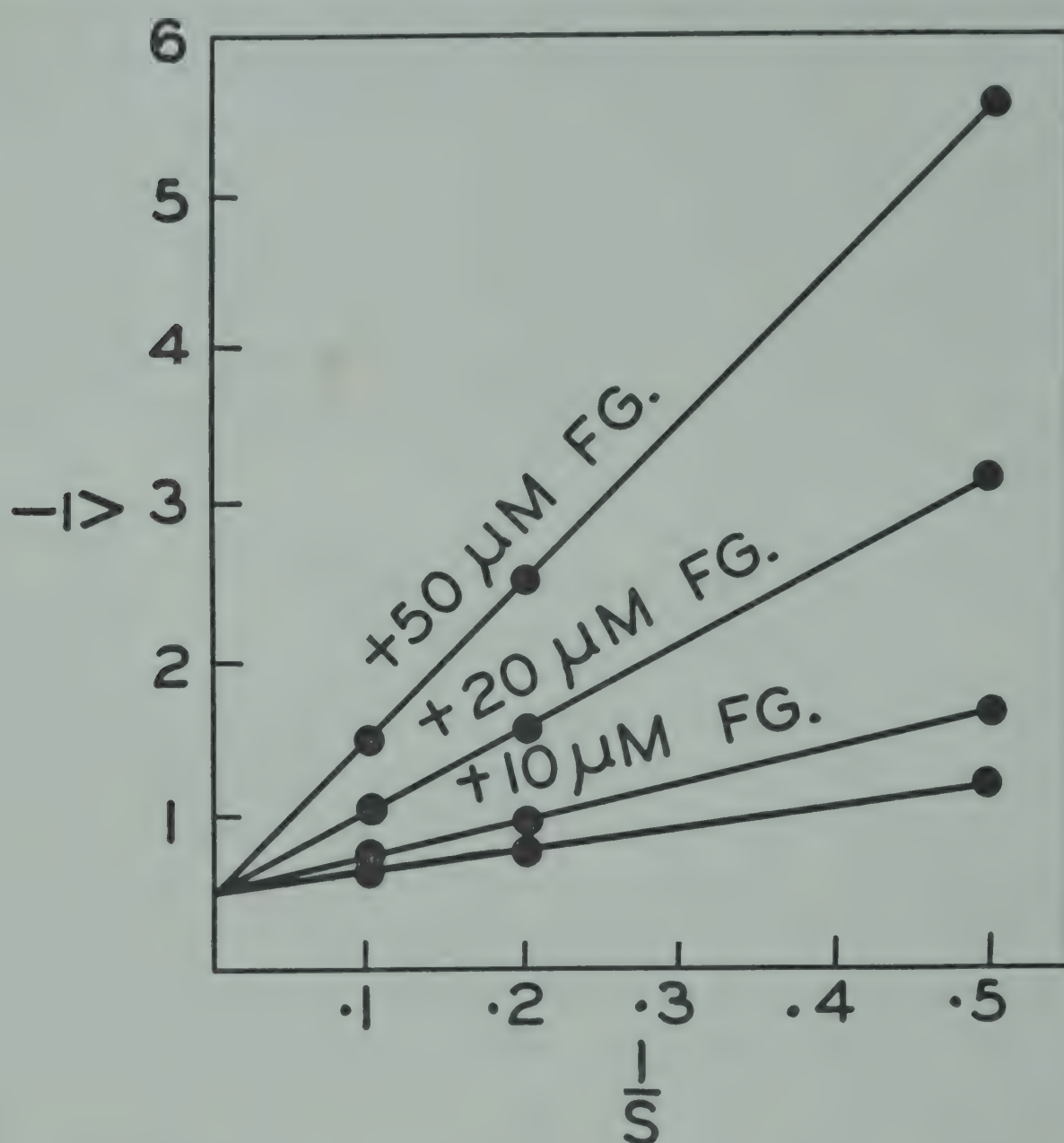


FIG. 3. Competitive inhibition of FG on CG for citrulline synthesis.

Final substrate concentrations expressed as micromoles per 2 ml.:  $\text{NaHCO}_3$ , 40; phosphate buffer, pH 7.4, 10;  $\text{NH}_4\text{Cl}$ , 20;  $\text{MgSO}_4$ , 20; ATP, 10; L-ornithine, 15; CG expressed in the abscissa in reciprocal micromoles; FG added as indicated in the figure, 3 mg. of enzyme preparation B (6) per tube. Final volume, 2 ml. Incubation at  $38^\circ\text{C}$ . for 15 min. The values obtained with CG and FG at 10, 20, and 50 micromole concentrations of the latter have been corrected for the "blank" values obtained with FG alone and are expressed in the figure. Velocities are given as reciprocals of the micromoles of citrulline synthesized under the experimental conditions of the figure. The lowest curve represents the values obtained from CG without added FG (S. Grisolia, unpub.).

First evidence of the reversibility of the ornithine  $\rightarrow$  citrulline reaction in mammalian tissues was obtained by Grisolia, Burris, and Cohen in exchange experiments with  $\text{N}^{15}$  (10); however, the extent of the exchange indicated the reverse reaction to be of a very

low order of magnitude in comparison with the forward reaction. Additional experiments to test the extent of reversibility of the reaction in mammalian tissues have been recently conducted as follows: we have studied the arsenolysis of citrulline in extracts of acetone powders containing activity for the synthesis of Compound X as well as for the formation of citrulline, and with a heated enzyme fraction from acetone powder extracts which is free of Enzyme I, but has about twice the specific activity for Enzyme II as the acetone powder extract. The results of this experiment, given in Table 6,

TABLE 6  
ARSENOLYSIS OF CITRULLINE

Experiment	Micromoles of Citrulline found at time		
	0	10 min.	20 min.
1	9.8	6.9	4.9
2	9.9	7.1	5.0

Concentration of components in micromoles per 1.5 ml.: citrulline, 10; sodium arsenate adjusted to pH 6.7, 75; citrate buffer, pH 6.7, 75; in experiment 1, 4.5 mg. protein, Enzyme II (Fraction E) (6) with an activity of 64.3  $\mu$ m citrulline/mg./10 min. were used; in experiment 2, 9.4 mg. protein, extract of washed rat liver residue acetone powder with an activity of 30.3  $\mu$ m citrulline/mg./10 min. Carbon dioxide and ammonia produced was equivalent to the citrulline decomposed. Incubation at 38° C. for the time indicated in minutes. (R. O. Marshall and S. Grisolia, unpub.).

show equal arsenolytic activity at equivalent concentrations of Enzyme II calculated for the forward reaction for both preparations. It appears probable, then, that the Enzyme II of our nomenclature is the enzyme system responsible for arsenolytic activity in mammals. If this is the case, these experiments confirm the deductions from the isotopic data (10), and again indicate that the back reaction, as such, may be of little importance in the mammal.

A preliminary survey of tissues for arsenolytic activity has shown that, as is the case for the forward reaction, there is practically no activity in extrahepatic tissues. This is further illustrated in Table 7.

It is logical to assume that the "ureidase" reaction in bacteria, or the reversal of the ornithine  $\rightarrow$  citrulline reaction in the mammal, involves the formation of a phosphate derivative of citrulline. Likewise in the citrulline  $\rightarrow$  arginine reaction the first stage might be the



formation of a citrulline phosphate compound (14). The possible existence of citrulline phosphate has long ago been reported (13).

TABLE 7  
ARSENOLYSIS OF CITRULLINE BY VARIOUS RABBIT TISSUE PREPARATIONS

Tissue	Citrulline	Tissue	Citrulline
Liver	3.9	Kidney	0.1
Cardiac Muscle	0.0	Brain	0.0
Skeletal Muscle	0.0		

Contents of incubation mixture in micromoles per 3 ml.: citrulline, 10; sodium arsenate (adjusted to pH 6.7), 100; citrate buffer, pH 6.8, 100. The following quantities in mg. of soluble protein from acetone powder water extracts of rabbit tissues were added: liver, 62.2; cardiac muscle, 14.8; skeletal muscle, 42; kidney, 47; brain, 6.2. Controls containing no sodium arsenate showed no citrulline decomposition. Incubation, 40 minutes at 38° C. Final volume, 3 ml. (R. O. Marshall and S. Grisolia, unpub.).

To put the possibility of citrulline phosphate formation to a more direct test, one of us (S. G.) collaborated sometime ago with Dr. S. Ratner. Purified Compound X was combined with Enzyme II, ornithine, and the rest of the purified enzymes and substrates, except for citrulline and ATP, necessary for the formation of arginine. It was not possible to demonstrate arginine formation in the absence of ATP. Therefore citrulline phosphate was not formed, or if it was it was not at the proper energy level for arginine formation to proceed. We recently conducted additional experiments in which we attempted to couple citrulline formation from Compound X and ornithine to ADP in the presence of hexokinase and glucose. It was not possible to show phosphate transfer. At the same time we explored the possibility of phosphate transfer with liver preparations in the presence of hexokinase and glucose from Compound X to ADP. Again no transfer was observed (Marshall, R. O. and Grisolia, S., unpub.).

These observations are of interest particularly in view of the observation that there are enzymes which decompose Compound X very rapidly. Some time ago one of the authors (S. G.) observed that skeletal and cardiac muscle homogenates, when added to mitochondrial preparations of rat liver, inhibited the reaction ornithine  $\rightarrow$  citrulline. The advances in the understanding of the mechanism

of the reaction have permitted us to investigate the action of tissue extracts upon Compound X. Fig. 4 shows the increase in the rate of decomposition of Compound X by muscle extracts. That the

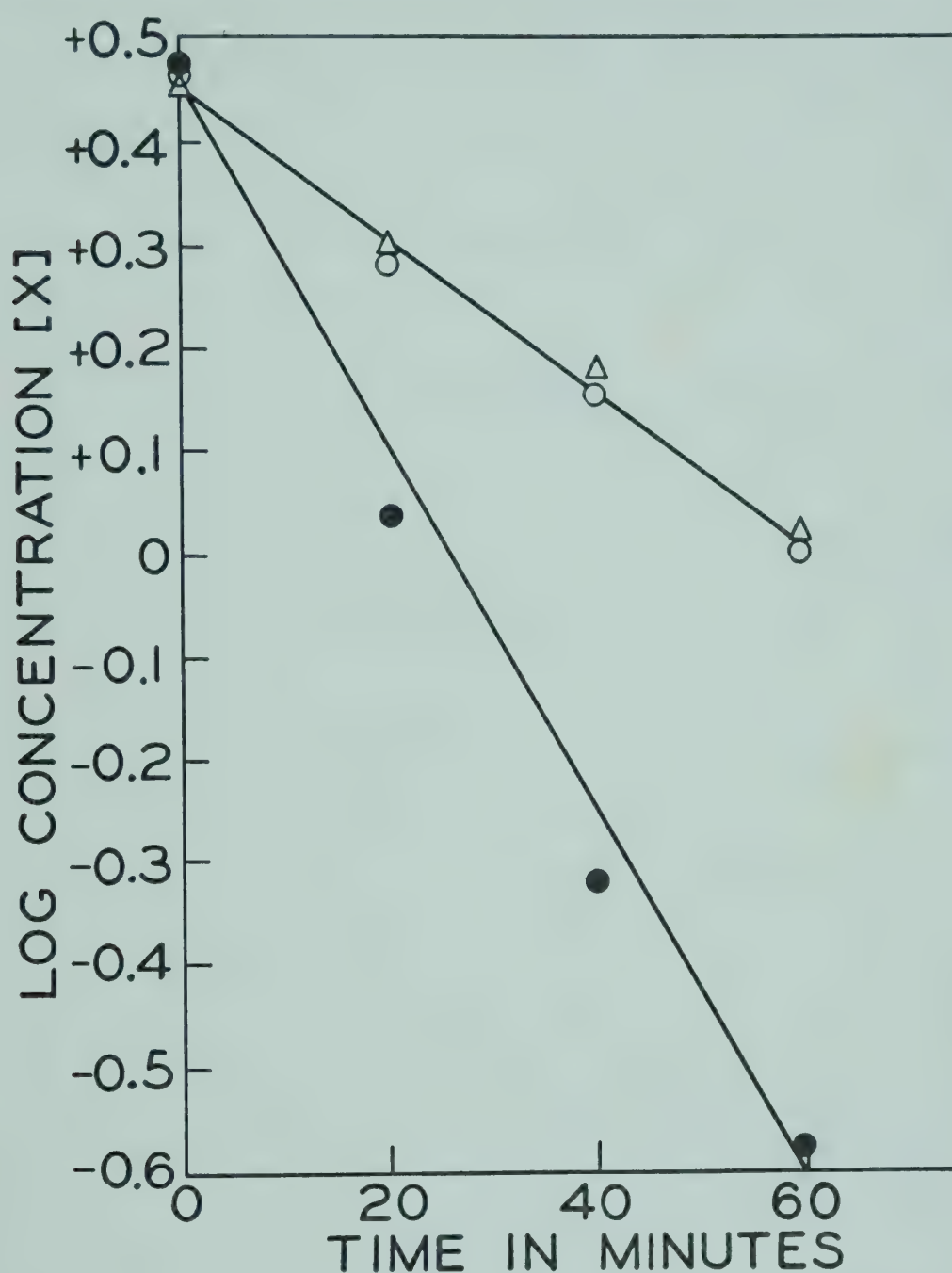


FIG. 4. The enzymatic decomposition of Compound X.

Each tube contained the following, expressed in micromoles per 2.5 ml.: tris-hydroxymethylaminomethane buffer, pH 7.4, 75; Compound X as potassium salt, 2.98;  $2000 \times g$  supernatant of a water extract of acetone-dried rabbit skeletal muscle, 26 mg. protein per tube. Temperature,  $38^{\circ}\text{C}$ . Experimental points marked with open triangles refer to spontaneous decomposition of Compound X. Experimental points marked with open circles refer to values obtained in the presence of heated (5 min. at  $100^{\circ}\text{C}$ .) muscle preparation. Experimental points marked with solid circles represent the values obtained in the presence of the unheated muscle preparation. Compound X was estimated in all cases as previously described (6). (Grisolia, S. and Marshall, R. O., 11).

decomposition is of an enzymatic nature is also indicated in the figure, since heated muscle preparations show no effect whatsoever on the nonenzymatic decomposition rate. Similar enzymatic decompo-



sition is also achieved by extracts from acetone dried preparations of other tissues. Referring the activity to brain on the basis of activity per milligram of protein, the following comparative values were obtained with rabbit tissue preparations, brain 100, cardiac muscle 17, skeletal muscle 12, liver 7, and kidney 7 (11). It is also possible that the enzyme described here may possess high activity when tested with other GD intermediates.

Although the intimate mechanism of the enzymatic decomposition of Compound X is unknown, it is reasonable to assume that it is due to dephosphorylation. If this is the case, it is most likely due to the action of a phosphatase and not to a transfer reaction. Regardless of the mechanism of the enzymatic decomposition of Compound X, the experiments reported here appear to be the first clue to a direct interference of sufficient magnitude to alter the efficiency of the urea cycle; thus it is conceivable that the phenomenon may be related to the specific dynamic action of proteins and related compounds. Since the synthesis of Compound X is an endergonic reaction, if the cell, for example, was not able to cope with the utilization of Compound X or related compounds due to the presence of active systems which will decompose the compound, a considerable leakage of high-energy phosphate might result.

These findings are schematically represented in Fig. 5. It is clear that there are still many questions to be answered for the understanding of the mechanism of citrulline synthesis and of the possible interrelations with other metabolic pathways.

Before closing it may be of interest to remember that whether carbamyl, acetyl, or a related compound proves to be the physiological catalytic agent for the biosynthesis of citrulline, had it not been for our erroneous earlier concept of a direct transcarbamylation reaction from CG, and our good luck, the elucidation of the mechanism of citrulline biosynthesis might have remained in the dark for a long time; for no one would reasonably think of adding catalytic quantities of an acetyl or carbamyl compound to support the synthesis of citrulline, *per se*, a catalytic agent in urea synthesis.

Dr. J. S. Fruton as well as other participants in this symposium have questioned the significance of the 100% purity for the tricyclohexylamine salt of Compound X given in Figure 1.

This criticism is well taken, particularly since we do not know as yet the correct structural formula of the intermediate. However, the purity has been estimated on the basis of a molecular weight of 607 for the tricyclohexylamine salt of Compound X which is com-

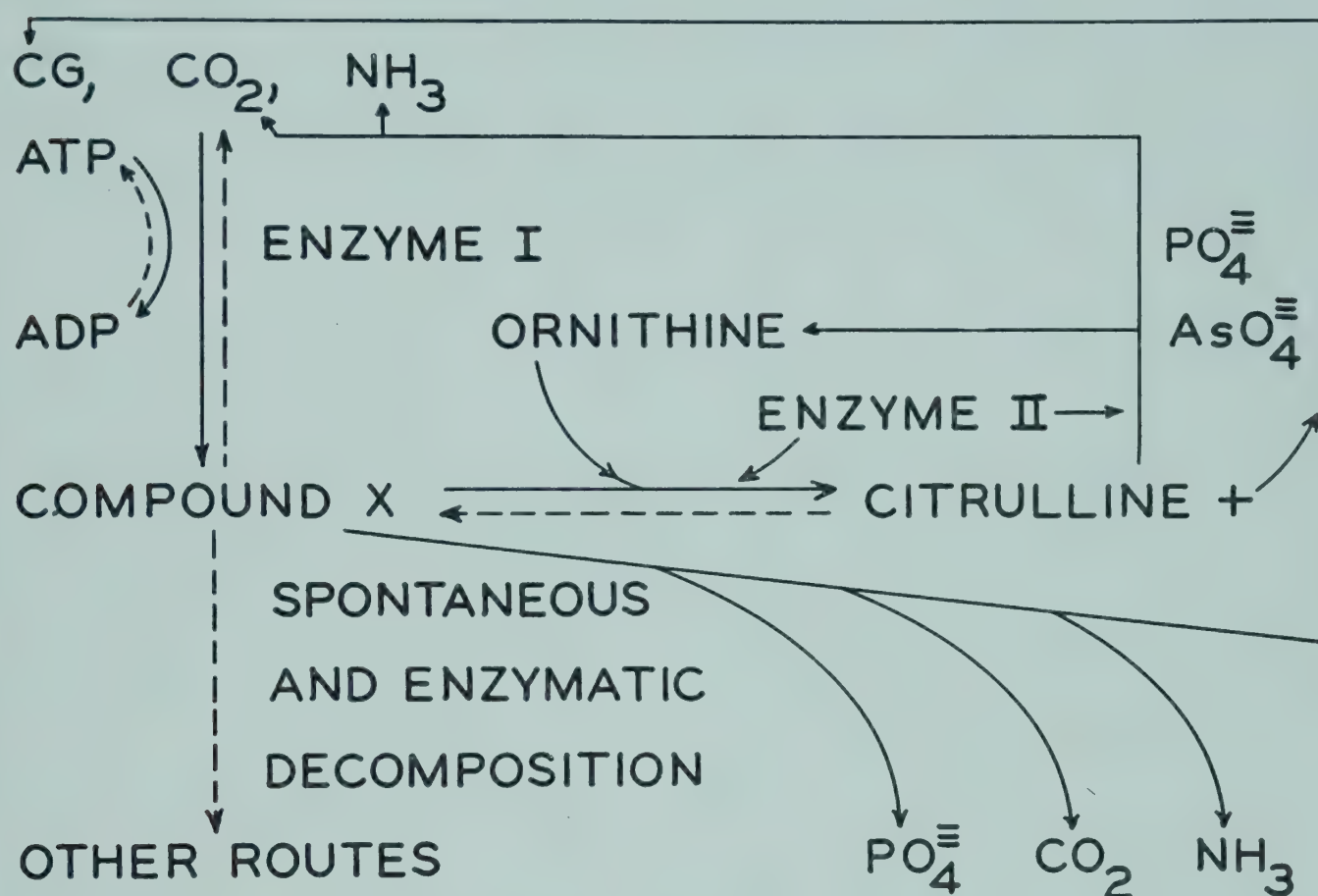


FIG. 5. Metabolism of Compound X and citrulline.

posed of equimolar quantities of CG, NH<sub>3</sub>, CO<sub>2</sub>, and phosphate. A typical analysis conducted by Mr. R. O. Marshall on a sample of the highest purity of Compound X called, for the purposes of this presentation, 100% in Figure 1 is as follows:

Wt. of sample (mg.)	Component Analyzed	Found ( $\mu$ M)	Theory ( $\mu$ M)
2.298	CHA *	11.50	11.40
2.418	NH <sub>3</sub>	4.15	4.02
5.00	Compound X (Enzymatically measured (6))	8.12	8.25
5.00	Phosphate	8.16	8.25

\* The estimation of cyclohexylamine has been carried out using a modification of the technique previously described for analysis of components of Compound X, with Dowex-2 columns followed by titration after distillation in a micro Kjeldahl apparatus.



It is apparent from these figures that we cannot be very far from absolute purity. Nevertheless the methods of analysis used are within 1-2% error.

However, using our relatively unspecific method of purification samples sometimes show near 1% ATP contamination. Again because of the lability of this compound caution must be used in keeping temperatures from 0 to  $-10^{\circ}$  (as soon as the use of organic solvents permits it) throughout to achieve successful purification.

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# METABOLIC RELATIONSHIP BETWEEN GLUTAMIC ACID, PROLINE, HYDROXYPROLINE, AND ORNITHINE

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THE OBVIOUS structural similarity between the amino acids glutamic acid, proline, hydroxyproline, and ornithine has, from the time of the identification of these compounds as important constituents of proteins, provoked speculation and research as to their possible metabolic interrelationships. Many lines of evidence have contributed to the present, still incomplete picture of the overall conversions which occur in various species and of the intimate steps over which these conversions take place. In a very abbreviated form the conversions under consideration are indicated in Fig. 1.

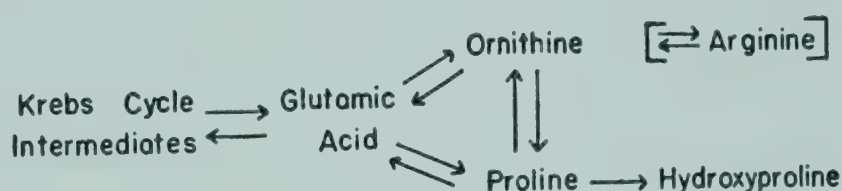


FIG. 1. Overall interconversions under consideration.

## OVERALL CONVERSIONS

*Glutamic acid.* The probable central role of glutamic acid in these interconversions has been evident from the time of the earliest crude nutrition studies up to the current investigations using mutant strains of microorganisms and isotopic labels. Abderhalden, in 1912, found that an alcohol-extracted casein hydrolysate, presumably free of proline and rich in glutamic acid, permitted normal growth of dogs. This led him to suggest that proline might be synthesized from glutamic acid (2). Dakin observed that glutamic acid, proline,



and ornithine were all glucogenic in diabetic animals, and he proposed that the three amino acids might be metabolically related (11). Classical nutrition studies from Rose's laboratory have given further indirect evidence for the conversion of glutamic acid into proline and ornithine. Growth rates of rats deprived of arginine, proline, hydroxyproline, and glutamic acid were significantly increased by the addition not only of arginine but also to a lesser extent of either proline or glutamic acid (49, 30). Direct evidence in the rat for the overall utilization of glutamic acid carbon for the synthesis of proline and ornithine, without regard to pathways, is seen in the finding of relatively high concentrations of  $C^{14}$  in proline and arginine isolated from rats fed glutamic acid- $C^{14}$  (31, 13).

There is considerable evidence that glutamic acid is the precursor of proline and ornithine in many bacteria and fungi (41, 9, 15, 48, 46, 39, 16, 44, 5, 4). Since Vogel has been responsible for many of the studies using *Escherichia coli* and *Neurospora crassa*, I shall review chiefly the animal studies and leave the details of the microbial phase of the problem to be covered by him in the succeeding paper, in order to avoid excessive duplication. It is sufficient here to say that normal operation of the Krebs cycle supplies the immediate precursor for the synthesis of glutamic acid (28), and that glutamic acid in turn acts as precursor for proline and ornithine (4, 6).

*Proline.* The overall biological conversion of proline into glutamic acid was established by tissue slice methods. The addition of proline to kidney and liver slice preparations resulted in an increase in oxygen uptake (7, 19) and amino nitrogen formation (47, 23). Weil-Malherbe and Krebs (47) isolated  $\alpha$ -ketoglutaric acid from the reaction products, and Neber (23) isolated glutamic acid itself. That proline is metabolized via glutamic acid in vivo was shown by the finding of stably bound deuterium as well as  $N^{15}$  in glutamic acid isolated from the proteins of rats which had been fed doubly labelled L-proline (38).

Stetten and Schoenheimer found, in these same experiments (38), that isolated ornithine also contained a significant amount of both D and  $N^{15}$ , demonstrating that the carbon chain of proline can be

converted into ornithine in the rat. The fact that proline can serve as a precursor for arginine helps to explain the definite though limited ability of the rat to synthesize arginine (33, 30).

*Hydroxyproline.* Hydroxyproline has been shown to be derived from proline in vivo (38). After L-proline- $N^{15}$ -D was fed to rats for 3 days, the hydroxyproline isolated from the carcasses was richer in isotope than was any other amino acid isolated, aside from proline itself. From a comparison of the respective ratios ( $N^{15}$ :D) in the hydroxyproline and the proline it appears that the conversion occurred with the loss or labilization of less than one half of the carbon-bound hydrogen. Thus the pathway of conversion must be a rela-

TABLE 1

Fed to Rats - 3 Days	Relative <sup>*</sup> Isotope Conc. in Isolated Amino Acids		
		<u><math>N^{15}</math></u>	<u>D</u>
Proline - $N^{15}$ - D	Proline	3.36	3.37
	Hydroxyproline	0.82	0.58
	Glutamic Acid	0.52	0.08
Hydroxyproline - $N^{15}$	Proline	0.05	
	Hydroxyproline	0.07	
	Glutamic Acid	0.27	
<sup>*</sup> Relative to 100 atom% $N^{15}$ and D in the compound fed			

L(—)-Proline- $N^{15}$ -D or DL-hydroxyproline- $N^{15}$  was mixed with the diet fed to rats for three days. Amino acids were isolated from the body proteins of the rats (38, 36).

tively direct one. The figures obtained indicated that about one quarter of all the carcass hydroxyproline had been produced from proline in three days in the rats studied (Table 1). Incidental confirmation of this extensive conversion was obtained from experiments in which ornithine- $N^{15}$  was fed to mice (37). The  $N^{15}$  concentration in the hydroxyproline isolated was about one-third as high as in the proline.

To investigate the utilization of hydroxyproline and the possible



reversibility of the conversion of proline to hydroxyproline, DL-hydroxyproline- $N^{15}$  was fed to rats for 3 days (36), and amino acids isolated. The results indicated that dietary hydroxyproline is handled in a very different fashion from proline and from all other amino acids previously studied. Whereas, under roughly comparable conditions, dietary tyrosine, leucine, lysine, histidine, glycine, etc., as well as proline, had been found to replace between 6 and 30 per cent of the corresponding amino acid in the body proteins of the rat in 3 days, less than 0.1 per cent of the hydroxyproline of the carcass had been replaced by dietary hydroxyproline in the same time. Much of this difference can be attributed to the peculiarly limited distribution of hydroxyproline in animals. Most of the hydroxyproline is in collagen, a protein which has been shown by Neuberger et al. (24) to have an exceedingly slow rate of turnover, whereas proline occurs also in proteins which are being regenerated more rapidly. This, however, is not sufficient to explain all of the results. A higher relative concentration of  $N^{15}$  was found in isolated hydroxyproline after labeled proline was fed than after hydroxyproline itself was fed (Table 1). These results have led us to conclude that most of the hydroxyproline occurring in body proteins is probably derived from proline that is already bound, presumably in peptide linkage, and not from free amino acid hydroxyproline. Free dietary hydroxyproline, although not appreciably utilized as such for protein synthesis, is extensively catabolized. Its  $N^{15}$  was found widely distributed in such amino acids as glutamic acid, aspartic acid, tyrosine, in the amidine group of arginine, and in high concentration in urinary urea and  $NH_3$ . In all of these compounds the  $N^{15}$  concentration was higher than in either the hydroxyproline or proline of the body proteins (36).

An unequivocal answer cannot be given from the evidence at hand as to whether any hydroxyproline can be reduced to proline *in vivo*. Proline isolated had an even lower isotope concentration than did the hydroxyproline. It is perfectly possible that all of this isotope came indirectly by synthesis of proline from breakdown products of the hydroxyproline fed, but the possibility of a small amount of direct reduction cannot be ruled out.



If any such reduction occurs, it is quantitatively unimportant. Whereas most, possibly all, of the tissue hydroxyproline comes from tissue proline, very little, if any, of the proline stems directly from hydroxyproline.

These findings are in accord with a number of observations in which the metabolism and oxidation rates of proline and hydroxyproline have been shown to differ (7, 47, 23, 14, 25). Nutrition studies have shown that proline, but not hydroxyproline, can replace a part of the arginine requirement of growing rats, and that hydroxyproline, when fed in relatively large amounts, may even inhibit growth (49).

*Ornithine.* The conversion of the carbon skeleton of ornithine into proline and glutamic acid was demonstrated by the finding of significant amounts of stably bound deuterium in proline and glutamic acid isolated from mice fed ornithine-D (29).

Studies of this sort, of course, give no indication of the detailed steps in the pathways over which these various interconversions occur, but a number of studies, particularly those using specifically labeled compounds or mutant strains of microorganisms, have yielded evidence as to the intermediate compounds and routes involved.

#### PATHWAYS OF INTERCONVERSION

In Fig. 2 is given a scheme of pathways and intermediates into which most of the accumulated evidence seems to fit. It is a pattern of reactions, evidence for which has been derived from a variety of biological systems, and it is possible that few systems exist in which all of these steps occur. Modifications and blocks at various steps certainly occur in many microorganisms and in mammals, and result in abolished or limited ability to synthesize one or another of the amino acids. A number of intermediates in addition to those indicated in Fig. 2 have at various times been considered.



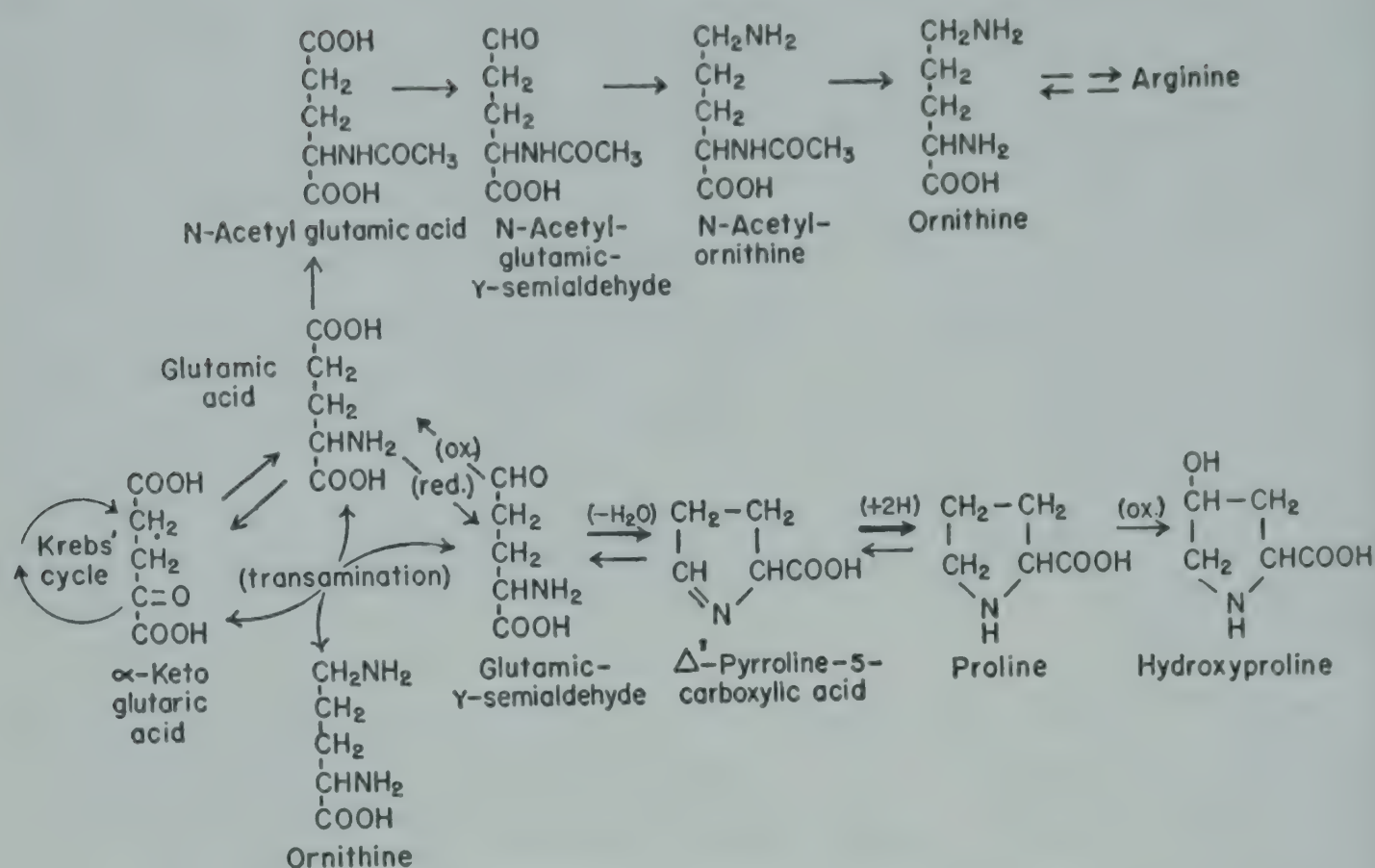


FIG. 2. Probable mechanisms for the biological interconversions of glutamic acid, proline, hydroxyproline, and ornithine.

*2-Pyrrolidone-5-carboxylic acid.* The earliest and most obvious possibility, 2-pyrrolidone-5-carboxylic acid, a glutamic acid anhydride, was suggested by Abderhalden as possibly occurring between glutamic acid and proline (1). When L(—)-pyrrolidone carboxylic acid was given by mouth or subcutaneously to rabbits it was rapidly and completely metabolized. D(+)-pyrrolidone carboxylic acid was excreted in the urine when DL-pyrrolidone carboxylic acid (3) or DL-glutamic acid (27) was given. Weil-Malherbe and Krebs (47) found that pyrrolidone carboxylic acid, in contrast to glutamic acid, proline, and hydroxyproline, had no effect on oxygen uptake, ammonia consumption, or production of amide or amino acid nitrogen in kidney slices, and concluded that it was not an intermediate. By the same kind of evidence Neber (23) showed that pyrrolidone carboxylic acid is not converted to proline or glutamic acid by liver or kidney slices. Pyrrolidone carboxylic acid will substitute for glutamic acid in *Micrococcus pyrogenes* var. *aureus* but will not replace proline in this organism or in a number of prolineless mutant strains of *E. coli* (17).

*$\alpha$ -Amino- $\delta$ -hydroxyvaleric acid.* A second compound which has been considered a possible intermediate is  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid. This would be the product of a hydrolytic splitting of the proline ring between the nitrogen and the  $\delta$ -C atom and would presumably be further oxidized at the  $\delta$ -C atom to give glutamic acid. Neber (23) observed that  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid, added to kidney slices, caused no utilization of ammonia for glutamine formation, such as was observed with both glutamic acid and proline, a fact that makes this compound very unlikely as an intermediate. Srb, Fincham, and Bonner (35) showed that  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid will, after a lag period, support growth of mutant strains of *Neurospora crassa* which require either ornithine, arginine, citrulline, or proline for growth, and they suggested that this might be the common intermediate between proline and ornithine. It is probable that in this organism the administered  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid was first converted into glutamic- $\gamma$ -semialdehyde before yielding either proline or ornithine. It was found that  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid could not be converted to proline in the mutant strains of *E. coli* studied by Vogel and Davis (46).

*$\alpha$ -Keto- $\delta$ -aminovaleric acid vs. glutamic- $\gamma$ -semialdehyde.* If the initial step from ornithine is pictured as an oxidative deamination or a transamination, one of two possible carbonyl compounds,  $\alpha$ -keto- $\delta$ -aminovaleric acid, or the  $\gamma$ -semialdehyde of glutamic acid would result, depending upon which of the amino groups of ornithine is lost. Likewise, initial dehydrogenation of proline on either side of the ring nitrogen atom, followed by hydrolytic opening of the ring, would result in formation of either one or the other of the same compounds. The alternative possibilities are indicated in Fig. 3.

$\alpha$ -Keto- $\delta$ -aminovaleric acid, as a possible intermediate, was suggested by the finding of Krebs (18) that the unnatural isomers D(+)-proline and D(—)-ornithine are both converted by preparations of D-amino acid oxidase of kidney into this  $\alpha$ -keto acid, which was isolated and characterized as its 2,4-dinitrophenylhydrazone. It was suggested that if the L-amino acids were metabolized like their D-isomers, the resulting  $\alpha$ -keto- $\delta$ -aminovaleric acid might undergo



oxidative deamination to the semialdehyde of  $\alpha$ -ketoglutaric acid, then further oxidation to  $\alpha$ -ketoglutaric acid, which secondarily, through transamination, would yield glutamic acid. Contradicting this alternative are the observations of Cedrangolo, Leone, and Guerritore (10) which indicate that in kidney or liver brei,  $\alpha$ -ketoglutaric acid formation is secondary to the formation of glutamic acid.

## ALTERNATIVE POSSIBILITIES

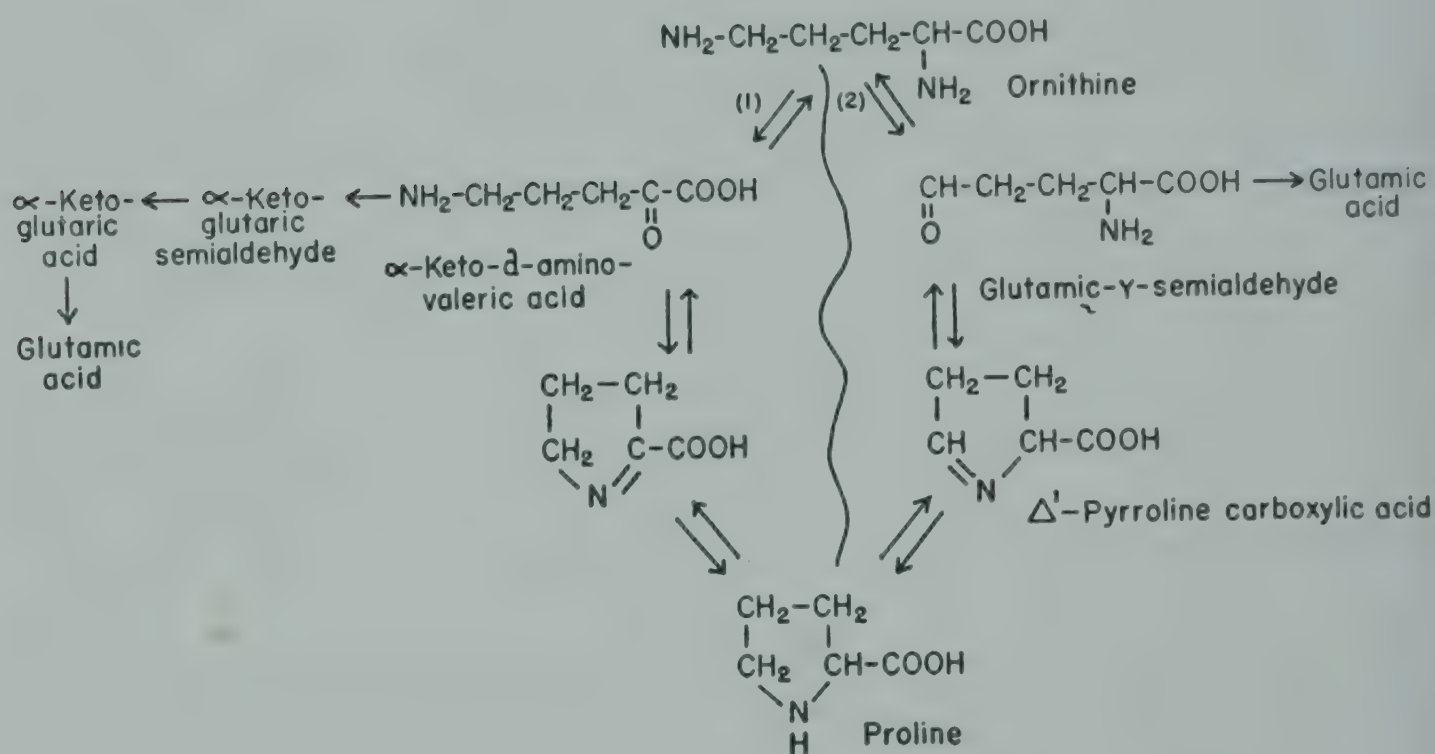


FIG. 3. Alternative routes for the interconversions of ornithine and proline.

$\alpha$ -Keto- $\delta$ -aminovaleric acid may be formed from L-proline as well as from D-proline (8). A product identical with that obtained by Krebs was isolated after the oxidation of L(—)-proline by kidney or liver L-amino acid oxidase preparations which were completely free of D-amino acid oxidase activity.

Taggart and Krakaur (40) found, on the other hand, that a preparation of washed insoluble particles of homogenized rabbit kidney was capable of oxidizing proline, hydroxyproline, and glutamic acid rapidly and completely under conditions which resulted in no disappearance of  $\alpha$ -keto- $\delta$ -aminovaleric acid. When oxidation of proline was incomplete, due to the omission of adenylic acid or magnesium ions from the system, a carbonyl compound accumulated which was isolated as a dinitrophenylhydrazone having the same

composition but different properties from the corresponding  $\alpha$ -keto- $\delta$ -aminovaleric acid derivative. This was presumably the hydrazone of the semialdehyde of glutamic acid. From a similar treatment of hydroxyproline a derivative was isolated having a composition in reasonable agreement with that expected for the 2-4-dinitrophenyl-osazone of  $\gamma$ -hydroxyglutamic semialdehyde. Thus the proline oxidase system is different in its action from that of the D-amino acid oxidase system of Krebs (18) or the L-amino acid oxidase system of Green (8). These results have recently been confirmed for proline (21) and for hydroxyproline (20). Lang and Schmidt found that a preparation of proline oxidase which gave glutamic acid semialdehyde from L-proline gave  $\alpha$ -keto- $\delta$ -aminovaleric acid from D-proline.

Despite the convincing evidence that  $\alpha$ -keto- $\delta$ -aminovaleric acid can be formed from proline or ornithine in mammalian systems, it is probable that it plays no significant role in the interconversions of L-proline, L-ornithine and L-glutamic acid in vivo, and that route 2, Fig. 3, more nearly represents the actual pathway of interconversion.

In an attempt to find out which of the possible alternative pathways from ornithine to proline is in fact operating in vivo, Stetten (37) studied the metabolism of two samples of DL-ornithine, one labeled with  $N^{15}$  specifically in the  $\alpha$ -amino group and the other with  $N^{15}$  exclusively in the  $\delta$ -amino group. In exactly comparable experiments these two compounds, mixed with a stock diet, were fed to mice for 9 days. In Table 2 are given some of the resulting analyses obtained on compounds isolated from these animals. Nitrogen from both amino groups of ornithine appeared in both proline and glutamic acid, but the relative values were very different. Proline derives considerably more of its nitrogen from the  $\alpha$ - than from the  $\delta$ -nitrogen of ornithine. Glutamic acid, on the other hand, derives more of its nitrogen from the  $\delta$ - than from the  $\alpha$ -amino group of ornithine. The  $N^{15}$  concentration was higher in glutamic acid than in proline when  $\delta$ - $N^{15}$  ornithine was given, but was higher in proline than in glutamic acid after  $\alpha$ - $N^{15}$  ornithine administration. These results indicate that in vivo the most important initial step in the oxidation of ornithine involves a selective transfer of the



TABLE 2

% of N in	Derived from $\delta$ -N of ornithine	Derived from $\alpha$ -N of ornithine
	%	%
Arginine		
Amidine N	0.56	0.42
$\alpha$ -Amino N	0.02	6.48
$\delta$ -Amino N	6.24	0.00
Glutamic acid	1.08	0.75
Aspartic acid	0.83	0.56
Proline	0.74	1.41
Hydroxyproline	0.29	0.46

DL-Ornithine- $\alpha$ -N<sup>15</sup> or DL-ornithine- $\delta$ -N<sup>15</sup> was mixed with the diet fed to mice for nine days. Amino acids were isolated from the body proteins of the mice (37).

$\delta$ -amino group to the labile pool of nitrogen exemplified by glutamic and aspartic acids. The remaining intermediate compound, presumed to be glutamic semialdehyde, probably undergoes immediate cyclization to a pyrroline carboxylic acid.  $\gamma$ - and  $\delta$ -amino aldehydes are known to undergo rapid spontaneous cyclization in neutral aqueous solution (32). The intermediate compounds may be converted by reduction to proline or by oxidation to glutamic acid. The extensive incorporation of the  $\alpha$ -amino group of ornithine into proline indicates that reduction to proline is the preferred route for glutamic semialdehyde and pyrroline carboxylic acids *in vivo*.

The arginine samples isolated were so degraded that separate analyses could be obtained on the different nitrogen atoms. The amidine, or urea precursor portion, of arginine derived nitrogen from both the  $\alpha$ - and  $\delta$ -amino groups of ornithine. In each case the N<sup>15</sup> in the isolated ornithine appeared almost exclusively in that position which had been labeled in the ornithine fed. The  $\delta$ -amino group of ornithine does not contribute any appreciable amount of nitrogen to the  $\alpha$ -amino group, nor does the  $\alpha$ -amino group contribute to the  $\delta$ -amino group. These results argue strongly against the significant occurrence of any cyclic intramolecular nitrogen shift in ornithine metabolism such as was suggested by Shemin and

Rittenberg (34). These authors noted that ornithine, isolated from rats at various times after  $N^{15}$ -glycine was fed, contained approximately the same concentration of  $N^{15}$  in the  $\alpha$ - and the  $\delta$ -amino groups. They speculated, on the basis of the previously known interconversions of proline and ornithine (29, 38), that, if both  $\alpha$ -keto- $\delta$ -aminovaleric acid and glutamic semialdehyde (Fig. 3) were intermediates, a cyclic process might result in which every N atom in the  $\delta$ -position of ornithine would pass through the  $\alpha$ -position. Such a process would have effected a redistribution of N between the two amino groups of ornithine. Since no transfer of N was detected, even after 9 days of feeding of specifically labeled ornithines (Table 2), a "cyclic intramolecular nitrogen shift" can be ruled out as playing any important part in these interconversions in vivo. The approximately equal labeling of the two amino groups of ornithine from an indifferent  $N^{15}$ -amino acid precursor can readily be explained by the assumption that the nitrogen of glutamic acid is the chief precursor of both amino groups of ornithine.

The use of a number of nutritionally deficient mutant strains of various microorganisms has proved of great value in tracing the pathways of synthesis and metabolism of these amino acids. Metabolic intermediates are sometimes found to accumulate when a mutant strain is unable to complete a normal chain of reactions. Thus isolation and identification of normal intermediates is facilitated. The findings (41, 9, 35) that mutant strains exist with a specific requirement for proline or for arginine, while in other strains the requirement may be met by glutamic acid, ornithine, arginine, or proline, led to the suggestion that in the parent strains of these microorganisms the interconversions of glutamic acid, proline, and ornithine occur through common intermediates by way of separate gene-controlled reactions (9, 35).

It was observed that a compound which accumulated during the growth of one proline-requiring mutant of *Escherichia coli* could be utilized for growth by another strain requiring proline, as well as for a third mutant which required either glutamic acid or proline (12). This proline precursor was found by Vogel and Davis (46) to be identical with synthetic  $\Delta^1$ -pyrroline-5-carboxylic acid, the cyclic



dehydration product arising spontaneously from glutamic- $\gamma$ -semialdehyde. Thus the pathway of proline synthesis in *E. coli* as well as in *Neurospora* (46, 16, 43) has been shown to proceed by way of the steps: glutamic acid  $\rightarrow$  glutamic- $\gamma$ -semialdehyde  $\rightarrow$   $\Delta^1$ -pyrroline-5-carboxylic acid  $\rightarrow$  proline.

Vogel has similarly found that an orthinine precursor is accumulated by one strain of *E. coli* and utilized by another. This compound has been isolated and identified by him as  $\alpha$ -N-acetylornithine (42). He has also demonstrated that in this organism the acetylornithine comes from acetylglutamic acid by way of acetylglutamic semialdehyde rather than by acetylation of glutamic semialdehyde (44, 45) (Fig. 2). Wild type *E. coli* contain a deacetylase capable of converting  $\alpha$ -N-acetylornithine into ornithine. The finding of Maas, Novelli, and Lipmann (22) that *E. coli* contain an enzyme which catalyzes the N-acetylation of glutamic acid also gives support to the pathway of ornithine synthesis in *E. coli* proposed by Vogel: glutamic acid  $\rightarrow$  N-acetylglutamic acid  $\rightarrow$  N-acetylglutamic- $\gamma$ -semialdehyde  $\rightarrow$   $\alpha$ -N-acetylornithine  $\rightarrow$  ornithine (see the following paper, by Vogel, for details).

The nature of the transamination reaction starting with ornithine has also been the subject of recent studies. Quastel and Witty (26) found ornithine transaminase action, distinct from glutamic transaminase, in rat liver homogenates. Amino nitrogen was transferred from ornithine to pyruvic, oxaloacetic, or  $\alpha$ -ketoglutaric acids. Since arginine could replace ornithine in these reactions only when arginase activity was also present, presumably the transamination proceeded from the  $\delta$ - rather than from the  $\alpha$ -amino group of ornithine. Fincham (16) prepared an extract of *Neurospora crassa* which was able to catalyze the transfer of the  $\delta$ -amino group of ornithine to  $\alpha$ -ketoglutaric acid with the formation of glutamic acid and glutamic- $\gamma$ -semialdehyde. Evidence that this reaction is reversible was obtained in experiments in which a small amount of ornithine was formed by *Neurospora* extracts from glutamic semialdehyde in the presence of added glutamic acid. Since extracts of mutant strains of *Neurospora* which require added ornithine were found to contain a normal amount of ornithine transaminase activity, it was concluded that



there must be some mechanism for ornithine synthesis other than the reversal of the ornithine transaminase reaction.

The finding that acetylglutamic acid is an intermediate in what may be the major route of ornithine biosynthesis in those organisms which do not require exogenous ornithine is a very interesting one. Presumably, blocking of the amino group of glutamic acid by acetylation permits reduction of the  $\gamma$ -carboxyl group and, with cyclization excluded, further conversion of the molecule to ornithine. Reduction of free glutamic acid to glutamic semialdehyde, on the other hand, leads preferentially to cyclization and conversion of the molecule to proline.

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# ISOTOPIC EQUILIBRATION BETWEEN THE CITRIC ACID CYCLE AND GLUTAMIC ACID \*

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WE SHALL discuss some interrelationships between glutamic acid and the citric acid cycle. The reactions involved have been so thoroughly discussed that it is not necessary to comment upon these here (1, 2). Our interest in these reactions arose in the course of the development of methods for study of the citric acid cycle and tributary reactions in tumors and other tissues in vivo. In the first of the methods used, the citric acid cycle was blocked with a specific metabolic inhibitor, such as fluoroacetate (3) or malonate (4), and the quantity of citrate or succinate that accumulated was determined. In the second method used, the isotope accumulating in pools of intermediates was determined after injection of a labeled intermediate such as acetate-1- $C^{14}$  (5-7) or pyruvate-2- $C^{14}$  (8). In early experiments, the rats were treated with malonate to induce accumulation of succinate which could be isolated and analyzed for radioactivity (5), but it was found that in both control and malonate-treated animals, as much as 70 per cent of the  $C^{14}$  in tissues was transferred to glutamate at short times after the tracer was injected (6, 7). The small dilution of isotope was apparent from the fact that the specific activity of glutamate of the heart and kidney was one-tenth that of the acetate-1- $C^{14}$  at 40 seconds after the injection of the tracer compound (7). The extent of labeling of glutamate varied for different tissues in terms of relative specific activity of the glutamate, percentage of the total isotope in the tissue found

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in glutamate, and the rates at which maximum specific activity of the glutamate was reached (7).

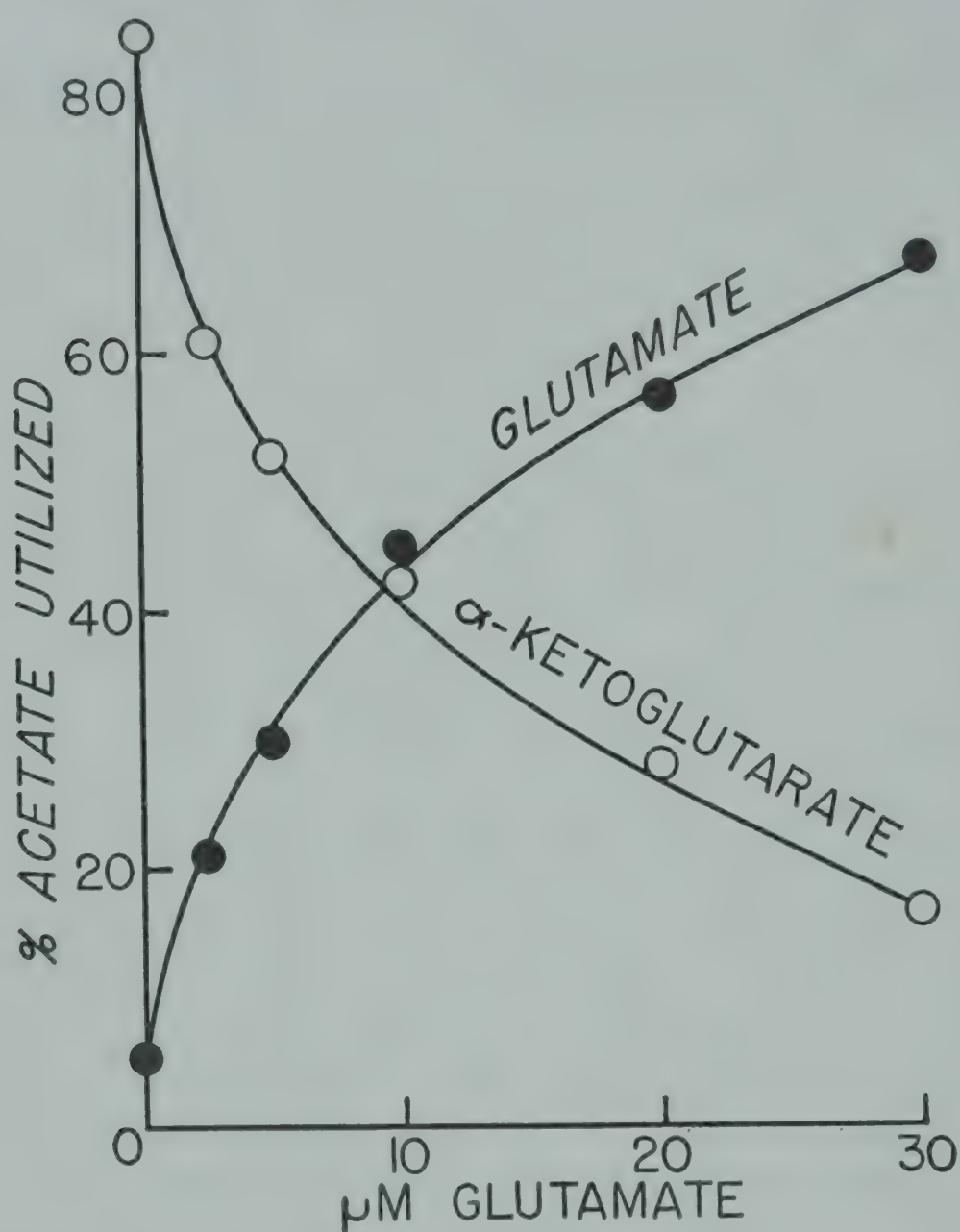


FIG. 1. EFFECT OF GLUTAMATE CONCENTRATION ON LABELING OF GLUTAMATE AND  $\alpha$ -KETOGLUTARATE

Reactions were carried out in Warburg flasks at 37.5° C. Flasks contained 0.5 ml. of 10% rat heart homogenate, 5  $\mu$ M potassium ATP, 20  $\mu$ M potassium pyruvate, 10  $\mu$ M potassium malate, 10  $\mu$ M  $\text{MgCl}_2$ , 200  $\mu$ M KCl and 40  $\mu$ M potassium phosphate buffer at pH 7.4. After adequate oxygen uptake was found in a 15-min. period of incubation, 0.05 mg. of acetate-1- $\text{C}^{14}$  was tipped into the main chamber from the sidearm. Incubation was continued for 30 min. Values are percentages of total acetate utilized and are averages for 3 flasks.

Because of the rapidity of entry of the isotope into the glutamate pool and claims of special pathways for succinate synthesis (9, 10), the reaction sequence involved in conversion of acetate carbons to glutamate carbons was studied further. Initial studies in vitro indi-

cated that the whole homogenate of heart transferred two-thirds of the isotope of the acetate-1-C<sup>14</sup> utilized to glutamate and another fourth to  $\alpha$ -ketoglutarate 20 minutes after the tracer was tipped into the main chamber of the flask from the sidearm (7). It was also found (Fig. 1) that as the concentration of glutamate in the medium increased, less isotope was found in  $\alpha$ -ketoglutarate and more in glutamate. This fact indicates that the extent of isotopic equilibration between  $\alpha$ -ketoglutarate and glutamate was dependent upon the relative concentrations of  $\alpha$ -ketoglutarate and glutamate.

Although Plaut (11) had concluded that the citric acid cycle was involved in acetate oxidation by heart mitochondria on the basis of labeling of  $\alpha$ -ketoglutarate, two other possible mechanisms were considered: first, a direct condensation of a 3-carbon unit with the acetyl moiety of acetyl-CoA and second, an exchange reaction involving the carboxyl of acetate. It should be stated at the outset that the evidence obtained thus far is consistent with the participation of the citric acid cycle in the labeling of  $\alpha$ -ketoglutarate and glutamate pools by heart and kidney.

With kidney homogenates, participation of the tricarboxylic acids in the metabolism of acetate-1-C<sup>14</sup> was readily demonstrated (Table 1). Addition of tricarboxylic acid resulted in extensive labeling of citrate and marked reduction in labeling of glutamate. Cis-aconitate and isocitrate were more effective than citrate in effecting labeling of the citrate pool. With heart homogenates, addition of tricarboxylic acids did not result in labeling of citrate or decrease in radioactivity of the glutamate and  $\alpha$ -ketoglutarate pool (Table 1). This paradox was pointed out by Plaut (11) for heart preparations, and similar effects have recently been discussed by Krampitz et al. (12, 13) in relation to studies on *Micrococcus lysodeikticus*. In the present studies, three procedures were used for the establishment of the citric acid cycle as the reaction sequence involved in the labeling of the glutamate pool in heart: (1) studies with fluoroacetate; (2) studies with various labeled compounds; and (3) degradation studies on glutamate. With kidney preparations, the experiments with fluoroacetate were in agreement with studies in which pools of



TABLE 1

EFFECT OF POOLS OF TRICARBOXYLIC ACIDS OF LABELING OF GLUTAMATE  
AND OF  
 $\alpha$ -KETOGLUTARATE BY HEART AND KIDNEY HOMOGENATES

Temperature of incubation, flask contents, and tipping of isotope were the same as in Fig. 1, with the exceptions that each flask contained 20  $\mu$ M potassium glutamate and 30  $\mu$ M potassium salt of tricarboxylic acid, as noted. Values represent percentages of acetate-1-C<sup>14</sup> utilized and are averages for three flasks.

Additions	Citrate	Kidney Homogenate		Glutamate + $\alpha$ -Ketoglutarate
		Glutamate	$\alpha$ -Ketoglutarate	
Control	15.4	62	21	83
+ Citrate	47.5	45	11	56
+ Cis-aconitate	80	19	1	20
+ Isocitrate	84	4	3	7
		Heart Homogenate		
		Glutamate	$\alpha$ -Ketoglutarate	
Control	0	71	18	89
+ Citrate	4.2	76	21	87
Control	0.6	48	42	90
+ Cis-Aconitate	1.5	49	41	90
Control	2.0	51	39	90
+ Isocitrate	2.5	59	31	90

TABLE 2

EFFECT OF FLUOROACETATE ON LABELING OF GLUTAMATE  
AND OF  
 $\alpha$ -KETOGLUTARATE IN HEART AND KIDNEY HOMOGENATES

Temperature of incubation and flask contents were the same as in Fig. 1 with the exceptions that each flask contained 20  $\mu$ M potassium glutamate, 0.05 mg. of acetate-1-C<sup>14</sup> containing 500,000 c.p.m. and 30  $\mu$ M sodium fluoroacetate where indicated. Experiment was carried out in triplicate, and flasks were incubated for 30 min. Values are percentages of acetate-1-C<sup>14</sup> utilized.

Additions	Citrate	Glutamate + $\alpha$ -Ketoglutarate
	<u>Kidney Homogenate</u>	
Control	15	82
+ fluoroacetate	87	6
	<u>Heart Homogenate</u>	
Control	0.5	89
+ fluoroacetate	62	32

tricarboxylic acids were used (Table 2). The isotope accumulated in citrate in the blocked system to an extent comparable to the accumulation of isotope in glutamate in the control. A similar but less complete effect was obtained with heart preparations. It should be noted that fluoroacetate exerts a marked depressant effect on oxidations and acetate utilization in heart homogenate systems.

The tracer compounds used in these studies were acetate-1-C<sup>14</sup>, pyruvate-2-C<sup>14</sup>, lactate-1-C<sup>14</sup> and bicarbonate-C<sup>14</sup>. Studies with these compounds (Table 3) indicated that label from pyruvate-2-C<sup>14</sup> and

TABLE 3

EFFECTIVENESS OF VARIOUS LABELED SUBSTRATES IN THE LABELING  
OF  
GLUTAMATE AND  $\alpha$ -KETOGLUTARATE BY RAT HEART HOMOGENATES

Flask contents, incubation temperature, and tipping of isotope were the same as in Fig. 1 with the exceptions that each flask contained 20  $\mu$ M potassium glutamate and the appropriate tracer compound. Values are percentages of total isotope added and are averages for 3 flasks.

Tracer Compound	Isotope in glutamate and $\alpha$ -Ketoglutarate
Acetate-1-C <sup>14</sup>	45.6
Lactate-1-C <sup>14</sup>	0.7
Pyruvate-2-C <sup>14</sup>	38.5
Bicarbonate-C <sup>14</sup>	1.1

acetate-1-C<sup>14</sup>, respectively, entered glutamate to the extent of 38.5 per cent and 45.6 per cent of the total isotope present, and of the acetate utilized, 78.5 per cent of the total was found in glutamate. On the other hand, 1.1 per cent of the isotope of bicarbonate-C<sup>14</sup> was found in glutamate and  $\alpha$ -ketoglutarate; this finding makes it unlikely that C<sup>14</sup>O<sub>2</sub> fixation plays a significant role in the labeling of glutamate. Only 6 per cent of the added lactate-1-C<sup>14</sup> was utilized, as measured by C<sup>14</sup>O<sub>2</sub> in the center well, but virtually none of the isotope was found in glutamate. Accordingly, a direct condensation of a C<sub>3</sub> and a C<sub>2</sub> fragment seemed less likely.

The final studies carried out on this point were degradation reactions on the isolated glutamate. The total isotope of the glutamate was determined after samples were plated directly, and the isotope in



C<sub>1</sub> was determined as BaCO<sub>3</sub> after treatment of the glutamate with chloramine T (14, 15). Activity in the remaining 4 carbons (C<sub>2</sub>—C<sub>5</sub>) was determined after the 2, 4-dinitrophenylhydrazone of the succinic semialdehyde was plated directly (15). The relative specific activity of C<sub>5</sub> and the whole molecule could be determined after trapping C<sup>14</sup>O<sub>2</sub> resulting from the Schmidt reaction on glutamate (16-18) while the isotope in C<sub>1</sub>—C<sub>4</sub> was determined by direct plating and combustion (19) of the dipicrate of  $\alpha$ ,  $\gamma$ -diaminobutyric acid, which was a product of the reaction. As indicated in Table 4, the isotope

TABLE 4

DISTRIBUTION OF ISOTOPE FROM ACETATE-1-C<sup>14</sup> IN GLUTAMATE

Glutamate was obtained by anion exchange chromatography of flask contents of experiments with kidney and heart homogenates in which additions were similar to those of Table 2. Values are averages of two experiments, and are percentages of the total isotope in the original glutamate.

Tissue	Carbons of Glutamate	
	C <sub>1</sub>	C <sub>5</sub>
Kidney	5	94
Heart	1	96

of glutamate isolated from both heart and kidney experiments was predominantly in C<sub>5</sub>. These data are consistent with the formation of asymmetrically labeled citrate (20) and the further sequence of reactions of the citric acid cycle to  $\alpha$ -ketoglutarate as the pathway of metabolism of acetate-1-C<sup>14</sup> in both heart and kidney.

One of the points of interest in the studies carried out on the metabolism of acetate-1-C<sup>14</sup> in vivo was the relatively low activity of liver of the male rat in acetate utilization and labeling of glutamate (7). The half-time of acetate utilization ranged from 6 to 20 seconds in most tissues, while the half-time of acetate utilization for liver was 48 seconds. Studies with whole homogenates indicated that the rate of utilization of acetate-1-C<sup>14</sup> by liver was one-twelfth that of kidney and one-eighth that of heart, respectively (Fig. 2). The mitochondrial fraction of liver exhibited the same relative order of magnitude in acetate-1-C<sup>14</sup> utilization in comparison with kidney mitochondria (Fig. 3). On the basis of acetate utilization and

labeling of glutamate, it would appear that the liver of the male rat is relatively inert toward acetate.

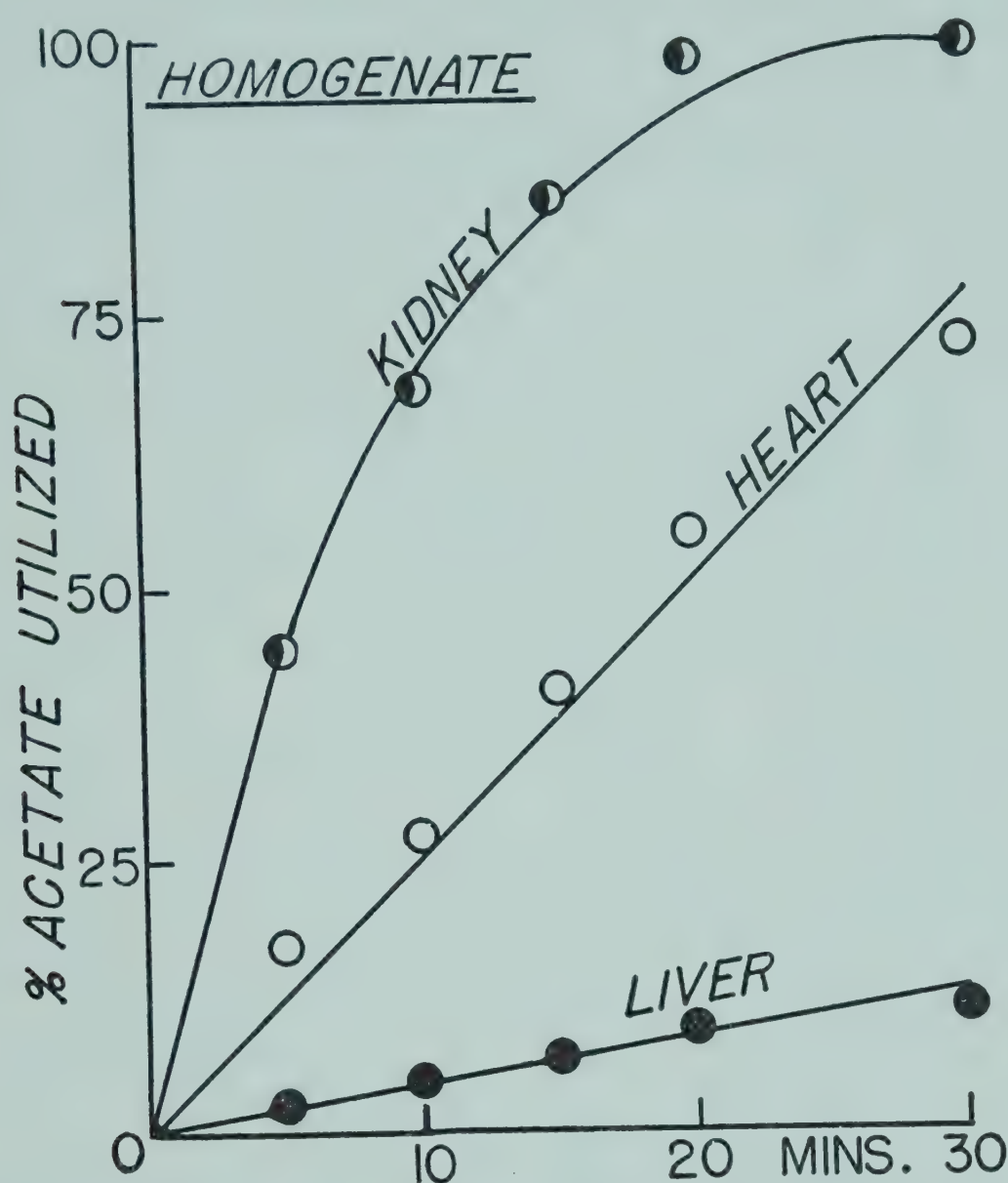


FIG. 2. Time course of acetate utilization by heart, kidney, and liver homogenates. Flask contents, incubation temperature, and tipping of isotope was the same as in Table 3. Values are percentages of total isotope added and are averages for 3 flasks.

Our primary interest in these reactions has been the further development of studies on the oxidative metabolism of tumors. It has been found that the half-time of utilization of acetate-1- $C^{14}$  in nontumor tissues ranged from 6 to 48 seconds, but for three transplantable tumors, the half-time for utilization of acetate-1- $C^{14}$  was 270 seconds. In the nontumor tissues, 33–76 per cent of the isotope was found primarily in glutamate, whereas in the tumors, only 4–6 per cent of the isotope was found in this substance and kinetic studies indicated that the tumor was not the likely source of the radioactive glutamate (7). From the data obtained, it was con-



cluded that the tumors studied were virtually incapable of metabolism of acetate-1- $C^{14}$ . In more recent studies (8) it was found that 90 per cent of the isotope of pyruvate-2- $C^{14}$  was transferred

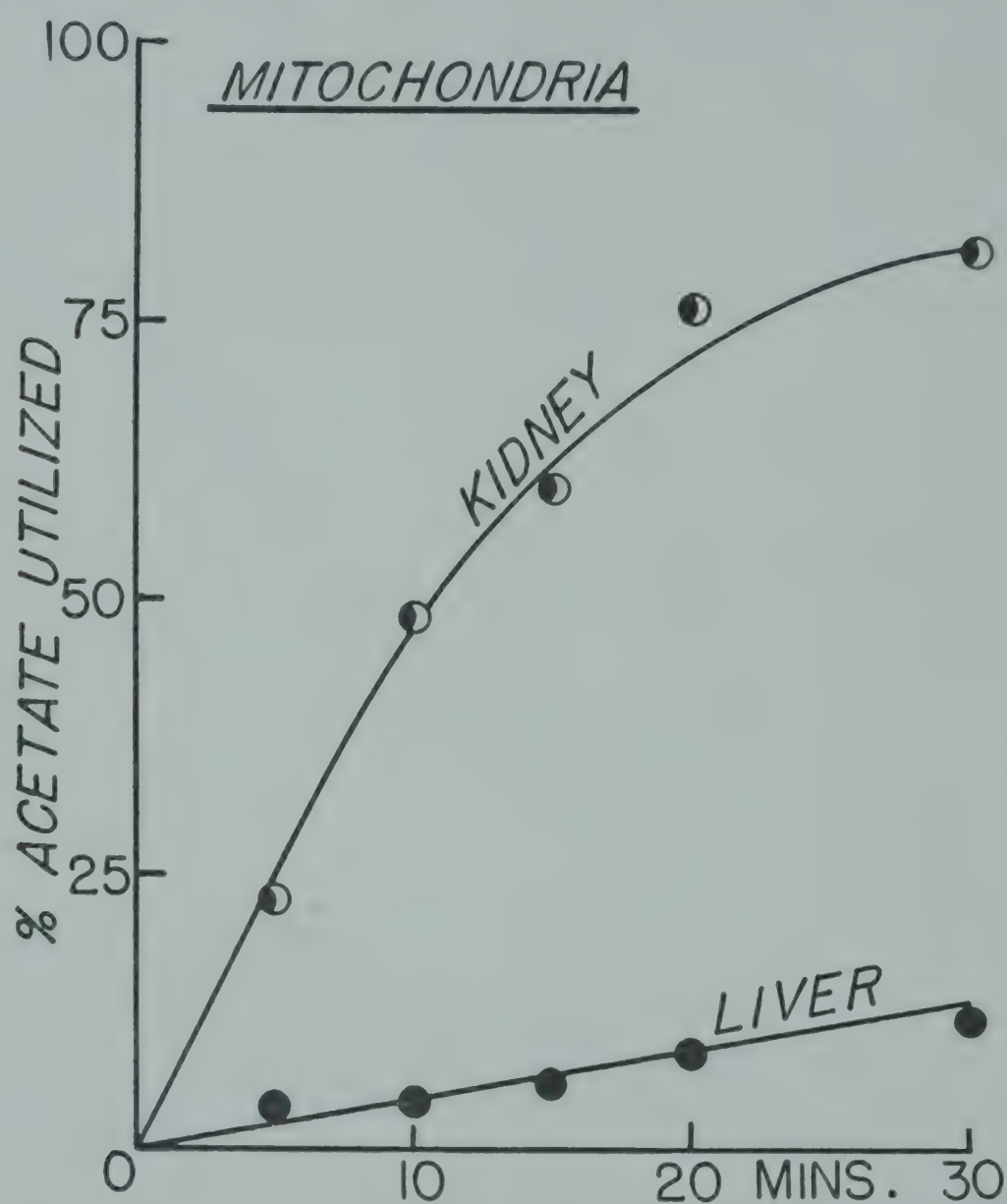


FIG. 3. Time course of acetate utilization by mitochondrial preparations of kidney and liver. Flask contents, incubation temperature, and tipping of the isotope was the same as in Table 3. Values are percentages of total isotope added and are averages for 3 flasks.

to lactate and 5 per cent of the isotope was transferred to the amino acids alanine, glutamate, and aspartate in the Walker 256 carcinosarcoma, whereas in nontumor tissues, 30–75 per cent of the isotope was found primarily in alanine and glutamate. Our present objective is the further investigation of these differences between tumors and nontumor tissues.

We wish to acknowledge the continued interest and helpful criticism of Dr. Ephraim Racker.

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# MECHANISMS OF ARGININE AND CITRULLINE BREAKDOWN IN MICROORGANISMS

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A DISCUSSION on the mechanisms of breakdown of arginine and citrulline should be prefaced, first of all, by pointing out the remarkable coincidence of simultaneous studies and discoveries, within the past two years, in at least four laboratories; or in at least eight laboratories, if the less detailed investigations be included. One can attempt to figure out what served as "the trigger reaction" to set off this research in so many places at once, but there appears to be no single definitive answer. Certainly the work of Ratner, Grisolia, and Cohen made the problems considerably easier to solve, but they had set up the guideposts for us quite some time before the recent studies. To one now granted hindsight and given the sense to see them, it is apparent that there were even earlier guideposts.

Out of the recent work of these several laboratories has come a unified pattern of the arginine to citrulline to ornithine reaction series, carried out by various bacteria. Some few genera, namely *Streptococcus*, *Pseudomonas*, and *Clostridium*, contain markedly greater amounts of the active enzymes than do other bacterial genera. We have detected only slight activity in all other bacteria tested, except for some *Staphylococcus* strains which were almost as active as the streptococci. One is perhaps tempted too much, when working in the area of the Krebs' urea cycle, to draw some sort of teleological or evolutionary meaning from the biological distribution of an enzyme pattern; however, one would be hard put to find three bacterial genera more dissimilar, from which to attempt to draw a general hypothesis. A much more thorough survey with many bacterial genera might well provide an understanding of the distribution of this enzymatic reaction series.

The overall reaction from arginine to ornithine was termed "arginine dihydrolase" some years ago (1); since there are two distinct steps in the reaction mechanism, it is preferable to consider them separately. The first step in the series is the conversion of arginine to citrulline and  $\text{NH}_3$ ; we have called the enzyme involved arginine desimidase, as proposed by Horn in 1933 (2). This enzyme is readily obtained in the cell-free state by ultrasonic oscillation of bacterial suspensions, or by water or buffer extraction of acetone-treated preparations (3). The latter method is actually preferable in the study of arginine desimidase, since these water extracts, by their complete inability to attack citrulline under the customary conditions of assay, permit quantitative accumulation of citrulline. The cell-free enzyme preparations are extremely stable when stored as lyophilized powder in the cold. The enzyme from *S. faecalis* has a pH optimum about 6.8, and is markedly inhibited by  $\text{Hg}^{++}$  and  $\text{Zn}^{++}$ , less strongly by  $\text{Cu}^{++}$  and  $\text{Mg}^{++}$  (4). Prolonged dialysis against distilled water results in essentially no inactivation. Since arginine desimidase is strongly inhibited by arsenite, by hydroxylamine, and by semicarbazide (4), we considered that these aldehyde-trapping compounds might be acting via inactivation of pyridoxal phosphate bound onto the enzyme. Several attempts were therefore made to demonstrate a function of pyridoxal phosphate in the reaction. Procedures such as inactivation by visible light, prolonged aging at room temperature, etc., did result in some small loss in enzyme activity, but the addition in vitro of pyridoxal phosphate (or of yeast extract or of kochsaft of untreated enzyme) did not restore activity. It might be added that tyrosine decarboxylase apoenzyme preparations of *S. faecalis*, that are stimulated by as little as millimicrogram quantities of pyridoxal phosphate, contain highly active arginine desimidase, but this activity is not increased by the addition of pyridoxal phosphate.

Thus far, we have found no cofactor requirements at all for the enzyme; nor does the addition of fumarate or malate increase the rate of reaction. We have not tested argininosuccinic acid with our arginine desimidase preparations. However, the arginine desi-



midase preparations from *S. faecalis* do not contain aspartase, and therefore the ammonia formed during the reaction cannot be derived via aspartic acid. In this regard, it should be noted that Walker and Myers (5) have found evidence in *Chlorella* for the competitive formation of argininosuccinate or citrulline, from arginine. Citrulline +  $\text{NH}_3$  formation in *Chlorella* preparations also occurs in the absence of aspartase.

The arginine desimidase reaction in cell-free extracts goes to completion, and no inhibition by the addition of citrulline has been observed. This reaction, arginine to citrulline +  $\text{NH}_3$ , appears to involve a distinctly different enzyme from the enzyme systems, previously discussed here, which catalyze the transformation of citrulline to arginine.

The second step in the reaction series, present primarily in the streptococci, the pseudomonads, and the clostridia, is the breakdown of citrulline to ornithine,  $\text{NH}_3$ , and  $\text{CO}_2$ , by an enzyme system called citrullinase, citrulline ureidase, or citrulline phosphorylase. This enzyme system took all of us who were working with it quite by surprise, one has little doubt, for it turned out to be capable of generating high-energy phosphate in the form of ATP (6, 7, 8, 9). The requirements for this reaction are as follows:

*First*, acetone-treated preparations, or cell-free extracts obtained by ultrasonic oscillation. Citrulline penetrates intact bacterial cells, particularly the streptococci, only very slowly. All three genera studied exhibit a rapid breakdown of arginine by intact cells, with relative accumulation of citrulline, particularly during the early and late periods of incubation. The rate of the citrullinase reaction is controlled by the concentration of the product, ornithine, as will be seen further in this paper, and the amount of citrulline accumulated by intact cells acting on arginine is thus influenced both by the rate of citrulline formation, and by the amount of ornithine produced by its decomposition.

*Second*, the presence of inorganic phosphate, magnesium ion, and AMP or ADP. The need for these compounds is more readily

apparent after dialysis of the enzyme preparations, as shown in Fig. 1. Prolonged dialysis of the preparations (120 hours in the experiments noted) almost completely destroys the citrullinase activity in the presence of added phosphate, AMP, and Mg. (9).

### Q<sub>CO<sub>2</sub></sub> OF CITRULLINASE

	<i>S. faecalis</i> Acetone Prep.		
	Undialyzed	48 Hours Dialyzed	120 Hours Dialyzed
No supplements	1.6	1.9	0
AMP	6.4	1.0	0
AMP + PO <sub>4</sub>	24.4	12.5	0
AMP + PO <sub>4</sub> + Mg	27.2	26.9	7.0
AsO <sub>4</sub>	28.0	—	31.1
AsO <sub>4</sub> + Mg	28.8	—	30.2

FIG. 1. Effect of supplementation on the rate of citrulline breakdown by *S. faecalis* acetone preparations.

The requirement of the enzyme system for these three substances, and the markedly lower response to ATP than to AMP or ADP, strongly suggested that inorganic phosphate was esterified, and that ATP was generated during the course of the reaction. It was then readily determined that per mole of citrulline broken down to ornithine, NH<sub>3</sub>, and CO<sub>2</sub>, one mole of inorganic phosphate was taken up, and one mole of labile phosphate formed. The formation of ATP was proved in various laboratories in different ways, and we cannot here take the time to describe the methods employed. The data are however unequivocal. Confirmation of the phenomenon, if one may be permitted the word, has been obtained by the several laboratories.

It has appeared most reasonable to assume that the reaction proceeds as follows: first, citrulline is phosphorylated by a kinase enzyme; second, through molecular rearrangement a high-energy bond is formed; and third, the phosphate is then transferred to AMP or ADP. However, a free citrulline phosphate has not yet been reported despite intensive search, and while the formation of such an ephemeral intermediate presents an attractive working



hypothesis, the data do not yet rule out the possibility that the reaction occurs entirely on the enzyme, i. e. that no free citrulline-phosphate exists as such.

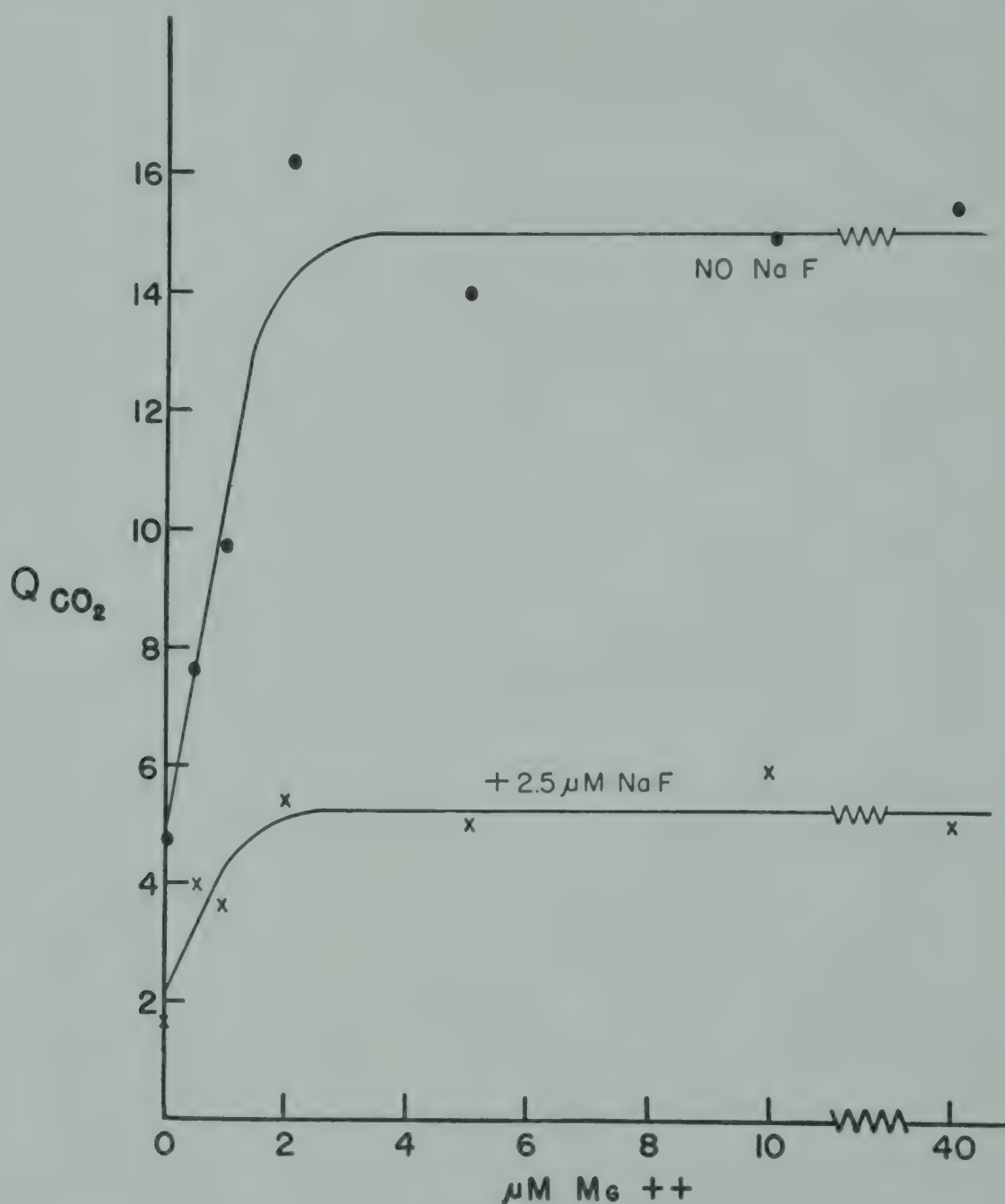


FIG. 2. Effect of  $\text{Mg}^{++}$  on fluoride inhibition of citrullinase.

Dialyzed *S. faecalis* acetone preparation (7 mg.) + 3  $\mu\text{M}$  AMP, 5  $\mu\text{M}$   $\text{PO}_4$ , 20  $\mu\text{M}$  DL-citrulline.

The requirement for  $\text{PO}_4$ , Mg, and AMP or ADP, can be replaced completely by the addition of arsenate (Fig. 1). The arsenolysis of citrulline proceeds rapidly even in preparations subjected to prolonged dialysis, and is completely uninfluenced by the presence or absence of Mg. This effect of arsenate on citrullinase is in agreement with the working hypothesis, since one would expect an arsenate ester to be subject to rapid spontaneous hydrolysis.

Carbamyl-L-glutamic acid exerts no stimulation of the reaction, under any experimental conditions, with even well-dialyzed preparations (10). Both phosphorolysis and arsenolysis are markedly inhibited by  $\text{Hg}^{++}$ , and by sodium fluoride. The inhibition by  $\text{Hg}^{++}$  can be reversed by either glutathione or BAL (9). The degree of fluoride inhibition is dependent on the system employed. With the

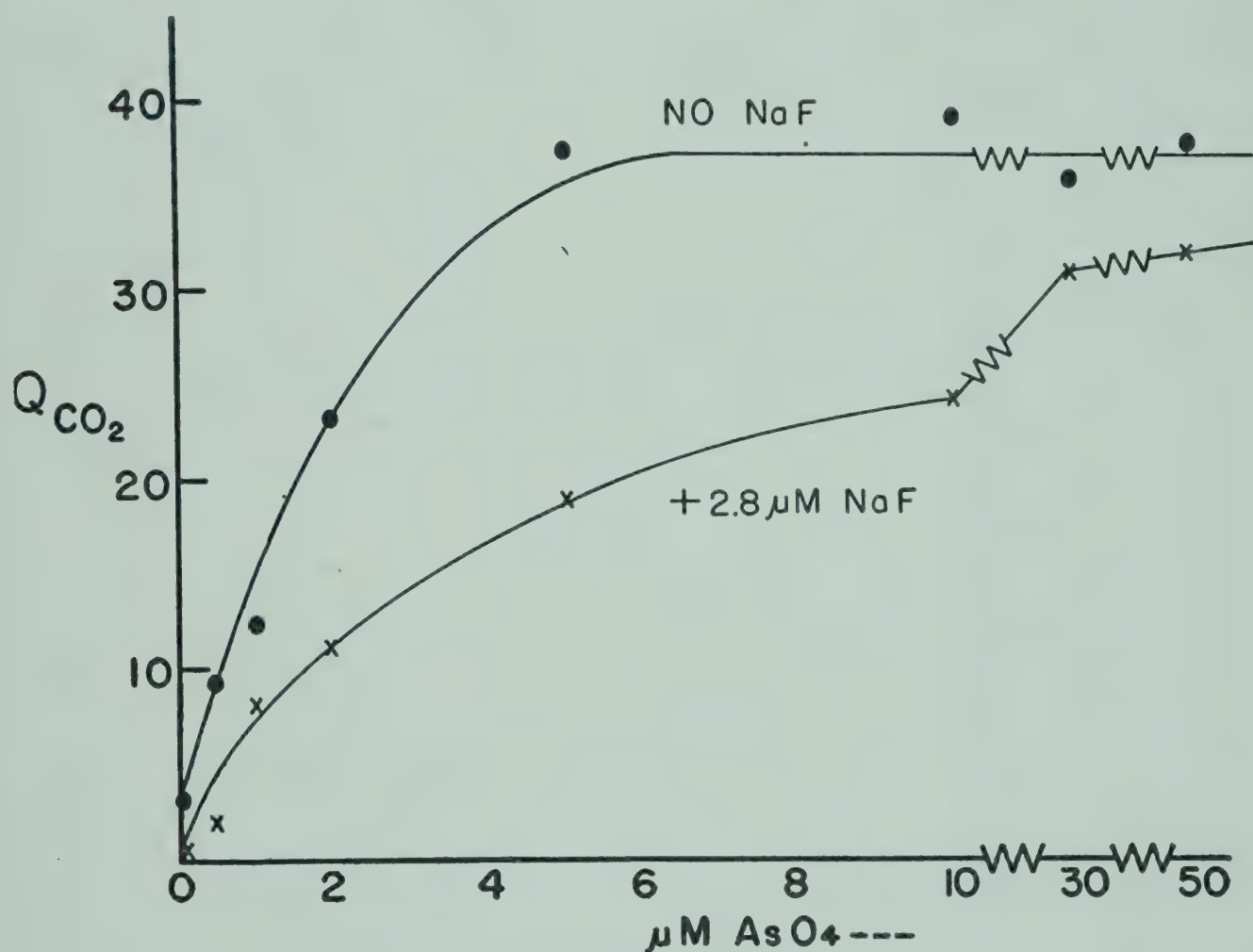


FIG. 3. Effect of  $\text{AsO}_4$  on fluoride inhibition of citrullinase.

Dialyzed *S. faecalis* acetone preparation (8 mg.) + 20  $\mu\text{M}$  DL-citrulline.

phosphate-AMP- $\text{Mg}^{++}$  system, concentrations of phosphate or  $\text{Mg}^{++}$  above the optimum do not affect the percentage of inhibition. The effect of fluoride at concentrations below the optimum, in this case the level of  $\text{Mg}^{++}$ , is shown in Fig. 2. The percentage of inhibition at 0.5  $\mu\text{M}$   $\text{Mg}^{++}$  is 46%, at 2  $\mu\text{M}$ , 67%; but it is quite clear that the increasing *per cent* inhibition results from the greater activity of the untreated enzyme, rather than from a further effect of  $\text{Mg}^{++}$  on the fluoride-treated preparation. Similar results are obtained at increasing levels of phosphate. That the inhibition by fluoride is not



due primarily to interference with  $Mg^{++}$  is shown by its interference with the arsenolysis system, in preparations subjected to prolonged dialysis. High concentrations of arsenate partially reverse the inhibition by fluoride, as seen in Fig. 3. The degree of reversal is dependent on the fluoride concentration as well.

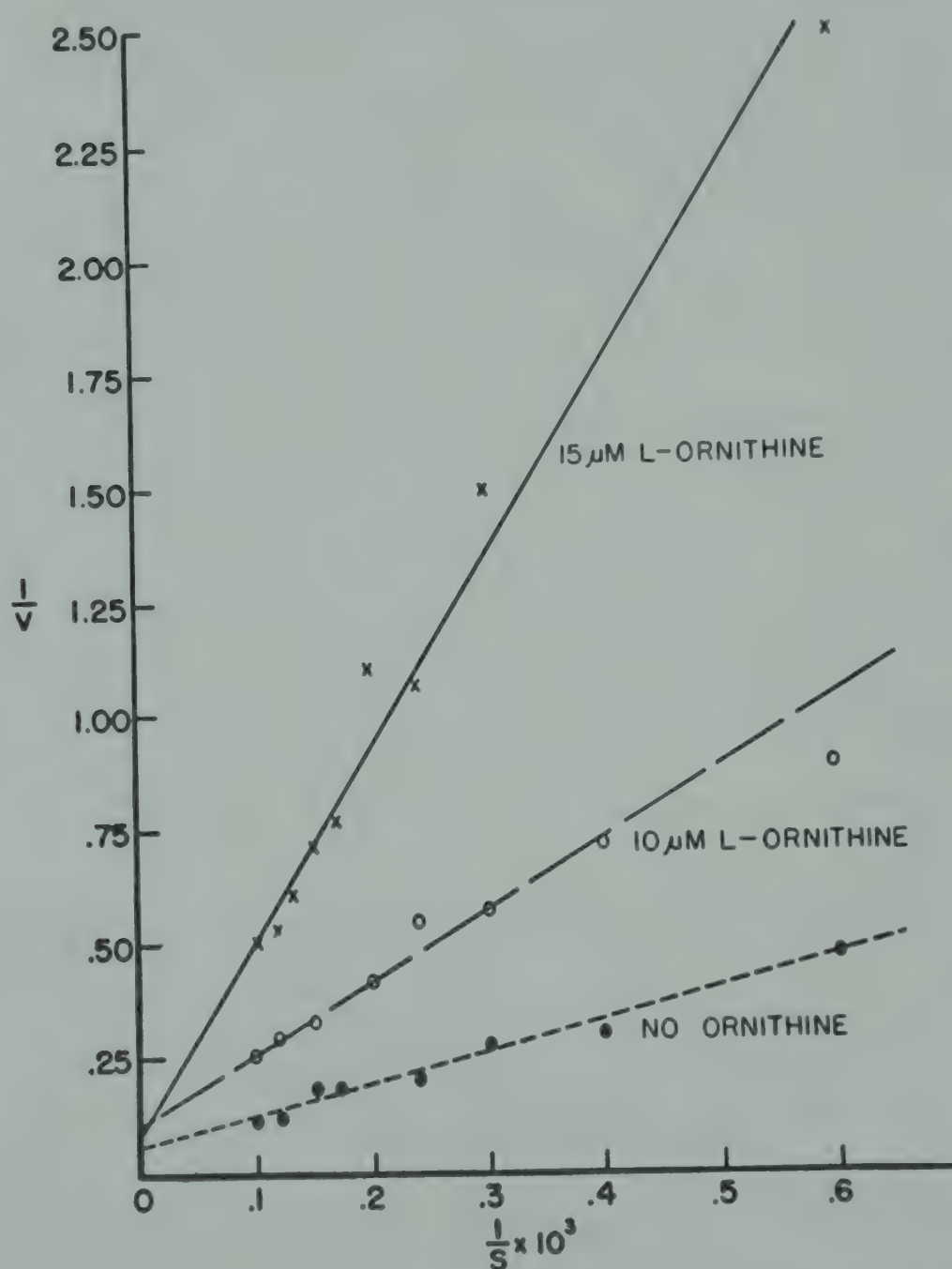


FIG. 4. Ornithine inhibition of citrulline arsenolysis. Cell-free extract, *P. aeruginosa*.

The citrullinase system is further sensitive to another mechanism of inhibition: the inhibition by ornithine. The rate of citrulline decomposition is linear only for the first 5 or 10 minutes of incubation, when one employs the customary 10 or 20  $\mu$ M of substrate and the phosphate system, and for about the first 15 to 20 minutes with the arsenate system. The reaction stops long before completion;

conversion values between 65% and 90% are obtained. This effect is due to the inhibitory action of the end-product, ornithine. Study of the kinetics of the inhibition was carried out with a cell-free extract of *Pseudomonas aeruginosa*, obtained by ultrasonic oscilla-

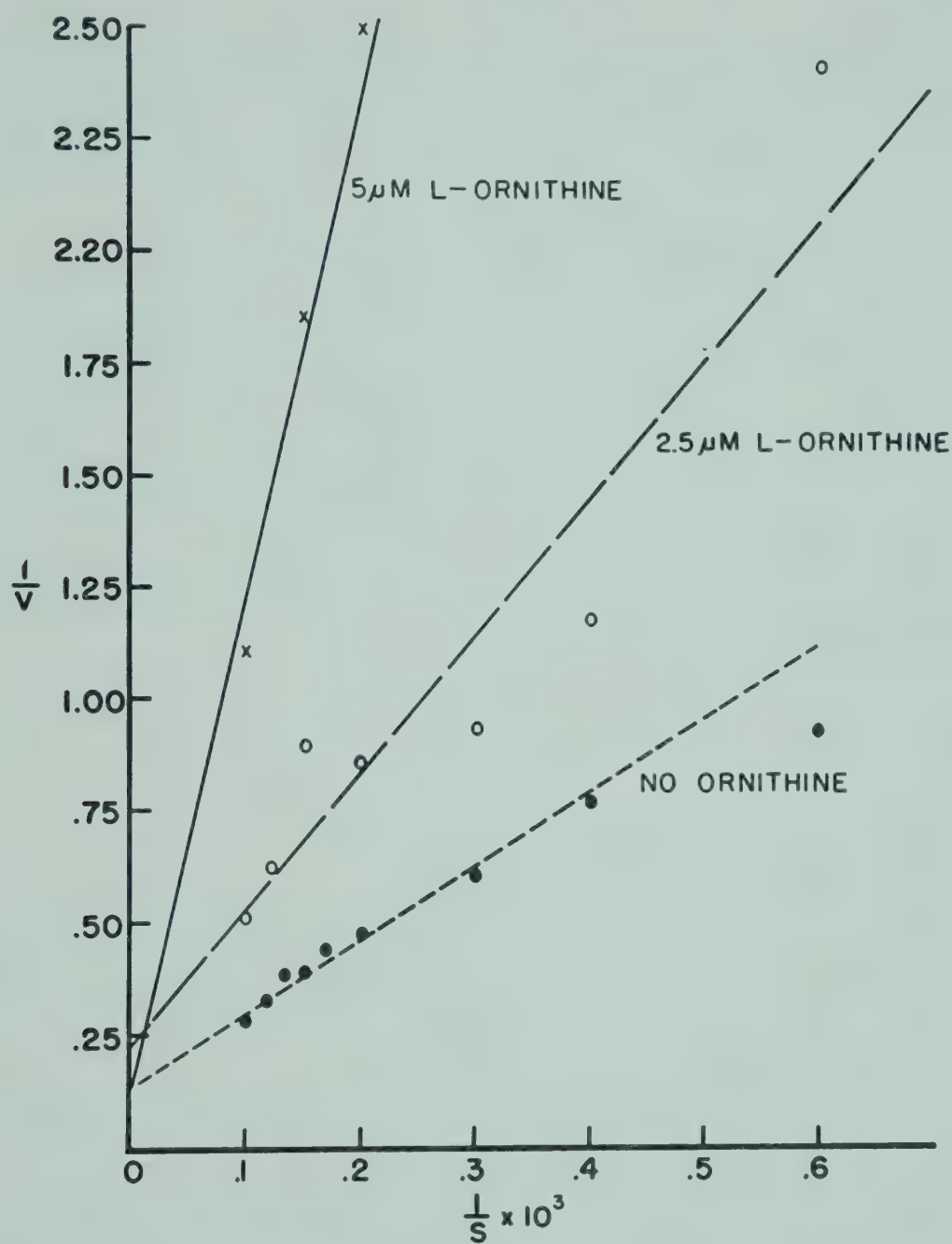


FIG. 5. Ornithine inhibition of citrulline phosphorolysis.  
Cell-free extract, *P. aeruginosa*.

tion, since our ultrasonicator does not produce as highly active extracts with the strain of *Streptococcus faecalis* employed in our other experiments. The inhibition by ornithine appears to be competitive, as shown by the Lineweaver-Burk type of plot illustrated in Fig. 4 for the arsenate system, with 10 and 15  $\mu$ M of L-ornithine. Similar results are shown in Fig. 5 for the phosphate system, but



here with 2.5 and 5  $\mu$ M of ornithine. It is readily apparent that the phosphate system is much more sensitive to the concentration of ornithine, which is in agreement with the more rapidly decreasing rates of citrulline decomposition in phosphate as compared to those in arsenate.

The rate of generation of high-energy phosphate by way of the decomposition of citrulline to  $\text{NH}_3$ ,  $\text{CO}_2$ , and ornithine is thus controlled in the cell not only by the concentration of the substrate and the cofactors required, but also by the concentration of ornithine. The usefulness of the citrullinase reaction in the cell economy must therefore be dependent as well on the enzymatic mechanisms for the utilization and transformation of ornithine. If one speculates further on the function of arginine desimidase for the cell, no other role is apparent than the formation of citrulline, one of the few substances thus far known whose metabolism is directly capable of generating high-energy phosphate.

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# METABOLISM OF ARGININE AND CITRULLINE BY BACTERIA \*

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## CONVERSION OF ARGININE TO ORNITHINE

THE PRODUCTION of ornithine from arginine by mixed putrefactive organisms was noted in early investigations by Ellinger (3) and Ackermann (1). The formation of ornithine was assumed to be the result of arginase and urease activity. In 1940 Hills (5) reported that gram-positive cocci, notably *Streptococcus*, liberate two moles of ammonia and one mole each of CO<sub>2</sub> and ornithine per mole of arginine metabolized. The reaction was depicted as hydrolytic in nature and was assumed to be catalyzed by one enzyme because neither urea nor citrulline was attacked. The enzyme, called arginine dihydrolase, was thus presumed to act in the manner of arginase and urease combined.

Washed whole cells of *Streptococcus lactis* release ammonia and

TABLE 1

DEGRADATION OF ARGININE BY CELLULAR SUSPENSIONS  
OF *Streptococcus lactis*

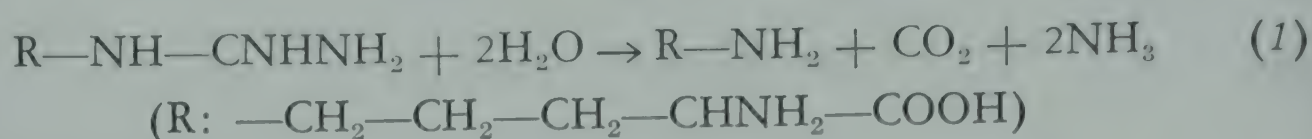
The reaction was conducted in Warburg vessels of conventional design. The main compartment of each flask contained 250 mg. of washed cells and 0.03 M. phosphate buffer, pH 6.4. Arginine was placed in one side-arm and 5% trichloroacetic acid in the other. Incubated for 10 min. at 30.4° C.

	Arginine $\mu$ M.	Citrulline $\mu$ M.	Ornithine $\mu$ M.	CO <sub>2</sub> $\mu$ M.	NH <sub>3</sub> $\mu$ M.
Initial	9.9	0	0.1	2.1	2.0
Final	1.9	0	8.1	10.6	18.1
$\Delta$	—8.0	0	+8.0	+7.9	+16.1

\* The work reported here was done at the Department of Bacteriology, Iowa State College, Ames, Iowa, and was supported by a grant from the U. S. Public Health Service.



CO<sub>2</sub> from L-arginine. Neither urea nor citrulline is metabolized. From the data presented in Table 1 it is evident that the degradation of arginine does not appear to involve citrulline and occurs according to the equation of Hills (5):



The arginine dihydrolase system has been reported in a variety of microorganisms (4, 20, 22, 14, 15, 24).

### CONVERSION OF ARGININE TO CITRULLINE

That citrulline may be an intermediate in the degradation of arginine was first indicated by Horn (6). After incubation of arginine with *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) citrulline was isolated from the reaction mixture. Horn named the enzyme responsible arginine desimidase. Tomota (23) reported similar results with the same organism. Sekine (18) and Akamatsu and Sekine (2) found that arginine was degraded to citrulline and ammonia by whole cells of *Streptococcus faecalis*. These authors proposed the name metarginase for the enzyme. Knivett (7) found that cell-free preparations or whole cells of *Streptococcus faecalis* which had been treated with acetone or cetyltrimethylammonium bromide attacked arginine. One mole of ammonia was liberated per mole of arginine degraded. Citrulline was identified chromatographically. Oginsky and Gehrig (12, 13) prepared cell-free extracts of *Streptococcus faecalis* and *Pseudomonas aeruginosa* and reported that arginine was degraded to equimolar quantities of ammonia and citrulline. Arginine desimidase, the name proposed by Horn (6), was adopted by Oginsky and Gehrig for the enzyme from *Streptococcus faecalis*. Slade (19) has reported similar observations with cell-free extracts of strain D10 of *Streptococcus faecalis*. Schmidt, Logan, and Tytell (17) obtained extracts of *Clostridium perfringens* (BP6K) which catalyzed the conversion of arginine to citrulline and ammonia. Roche and Lacombe (16) found that extracts of baker's yeast also metabolize arginine to citrulline. The name arginine desiminase for the enzyme was suggested by these authors.

Dialyzed cell-free extracts of *Streptococcus lactis* metabolize L-arginine to equimolar amounts of citrulline and ammonia, as indicated in Table 2. The extracts are inactive on citrulline when

TABLE 2

## CHEMICAL BALANCE OF PRODUCTS

The test system consisted of 0.12 M. acetate buffer pH 5.7, 0.02 M. sodium fluoride, enzyme, substrate, and water to a total volume of 2.0 ml.; gas, air. Incubated 1 hr. at 30° C.

	Arginine $\mu$ M.	Citrulline $\mu$ M.	Ammonia $\mu$ M.
Initial	10.1	0.0	1.1
Final	0.2	10.1	11.1
$\Delta$	—9.9	+10.1	+10.1

tested in acetate, maleate, or phthalate buffers. Fluoride may be used to inhibit the slight activity on citrulline in the presence of phosphate. The pH optimum for both ammonia and citrulline production extends from pH 5.4 to pH 5.8. Oginsky and Gehrig (13) reported that pH 6.8 is optimum for citrulline production by cell-free extracts of *Streptococcus faecalis*. Slade (19) did not present data showing the effect of pH upon the degradation of arginine to citrulline by extracts of *Streptococcus faecalis*, but made experiments at pH 5.8. Schmidt, Logan, and Tytell (17) found more ammonia liberated from arginine at pH 5.5 than at either pH 6.8 or pH 7.2 with extracts of *Clostridium perfringens* (BP6K). The maximum rate of arginine degradation by extracts of baker's yeast occurs at pH 6.5 (16).

Oginsky and Gehrig (13) reported that semicarbazide, hydroxylamine, and arsenite inhibit the liberation of citrulline or ammonia from arginine by extracts of *Streptococcus faecalis*. No inhibition by semicarbazide, hydroxylamine, iodoacetate, or potassium azide was noted with extracts of *Streptococcus lactis*; arsenite was found to be inhibitory (Table 3). Roche and Lacombe (16) have found that azide is inhibitory to plasmolysis sap but not to alcohol fractionated extracts of acetone-dried baker's yeast.

Dialyzed extracts of *Streptococcus lactis* are highly specific for



arginine. No ammonia is produced from any other amino acid tested nor from creatine, creatinine, glycoxyamine, urea, semicarbazide, carbamyl-DL-aspartic acid or carbamyl-L-glutamic acid. The extracts are

TABLE 3

## EFFECT OF INHIBITORS

The test system is described in Table 2; 0.025 M. L-arginine. Incubated for 20 min. at 30° C.

No.	Inhibitor	Ammonia μg.	Inhibition Per Cent
1.	None .....	244	0
	0.018 M. semicarbazide... ..	243	< 1
	0.018 M. hydroxylamine.....	244	0
	0.02 M. NaHAsO <sub>3</sub> .....	42	83
	0.0025 M. NaAsO <sub>2</sub> .....	80	68
	0.005 M. NaAsO <sub>2</sub> .....	65	74
	0.01 M. NaAsO <sub>2</sub> .....	44	83
	0.02 M. NaAsO <sub>2</sub> .....	32	87
2.	None .....	181	0
	0.02 M. iodoacetate.....	180	< 1
3.	None .....	218	0
	0.03 M. potassium azide.....	218	0

useful for the quantitative degradation of the guanido group of arginine to a carbamido group in the presence of amino acids, guanido and carbamido compounds.

The mechanism whereby arginine is degraded to citrulline is unknown. Inasmuch as cell-free extracts are not rendered inactive by prolonged dialysis, it does not appear likely that the bacterial decomposition of arginine is the reverse of the arginine-synthesizing reaction found in mammalian liver.

## CONVERSION OF CITRULLINE TO ORNITHINE

Sekine (18) and Akamatsu and Sekine (2) stated that citrulline is decomposed with the production of ammonia by whole cells of *Streptococcus faecalis*. They named the enzyme responsible citrullinase. Knivett (7) found that the citrulline was attacked very slowly by intact cells of *Streptococcus faecalis* and not at all by cells treated with acetone or cetyltrimethylammonium bromide. Upon addition of large amounts of ATP, cells which had been treated

with cetyltrimethylammonium bromide metabolized citrulline to ornithine, ammonia, and  $\text{CO}_2$ . Oginsky and Gehrig (12) reported that acetone-dried cells of *Streptococcus faecalis* rapidly formed ammonia and  $\text{CO}_2$  from citrulline, whereas untreated cells or cell-free extracts were inactive. Schmidt, Logan, and Tytell (17) reported that citrulline was attacked by untreated cells of *Clostridium perfringens* (BP6K). Cell-free preparations were found to be inactive. Korzenovsky and Werkman (9, 10) were the first to describe the preparation of cell-free extracts capable of converting citrulline to equimolar quantities of ornithine,  $\text{CO}_2$ , and ammonia, as shown in Table 4. The extracts were prepared by sonic disinte-

TABLE 4

## CHEMICAL BALANCE OF PRODUCTS

The test system consisted of 0.2 phosphate buffer, pH 6.2-6.3, 0.005 M. ADP, 0.005 M.  $\text{Mg}^{++}$ , enzyme, and water to a final volume of 1.0 ml.; gas, air. Incubation time, 1 hr.

	Citrulline $\mu\text{M.}$	Ornithine $\mu\text{M.}$	$\text{NH}_3$ $\mu\text{M.}$	$\text{CO}_2$ $\mu\text{M.}$
Initial	4.9	0	0.5	0.3
Final	0.1	4.8	5.4	5.1
$\Delta$	-4.8	+4.8	+4.9	+4.8

From Korzenovsky and Werkman (10).

gration of *Streptococcus lactis*. The components of the enzyme system are given in Table 5. It is evident that the conversion of citrulline to ornithine requires orthophosphate,  $\text{Mg}^{++}$ , and ADP. In the presence of arsenate neither  $\text{Mg}^{++}$  nor ADP is necessary. These two components are thus linked to the function of orthophosphate in the degradation of citrulline. Kinetic studies (10) indicated that orthophosphate is required in substrate concentrations for maximum activity, a fact suggesting that inorganic phosphate enters the reaction stoichiometrically. This idea was strengthened by finding that the concentration of orthophosphate decreases during the conversion of citrulline to ornithine. Although a decrease in orthophosphate is readily shown in the absence of hexokinase, glucose, and iodoacetate, the addition of these components greatly enhances this effect. Inasmuch as extracts of *Streptococcus lactis*



contain enzymes which destroy acid-labile phosphate bonds, it is to be expected that the extent of esterification, measured as a decrease

TABLE 5  
COMPONENTS OF ENZYME SYSTEM

The test system consisted of 0.2 M. phosphate or arsenate buffer, pH 6.2-6.3, 0.05 M. L-citrulline, 0.005 M. ADP and Mg<sup>++</sup> when used, enzyme, and water to a final volume of 1.0 ml.

Components	Specific Activity	
	Undialyzed	Dialyzed
Phosphate, ADP, Mg <sup>++</sup> .....	16.8	8.1
Phosphate, ADP.....	7.3	1.4
Phosphate, Mg <sup>++</sup> .....	3.3	1.0
Phosphate .....	1.8	0.4
*ADP, Mg <sup>++</sup> .....	—	1.4
Arsenate .....	—	125.5
Arsenate, ADP, Mg <sup>++</sup> .....	—	122.1

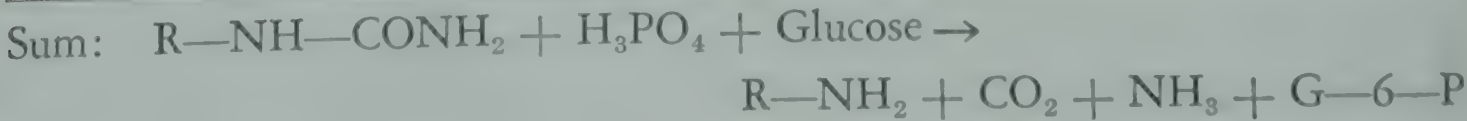
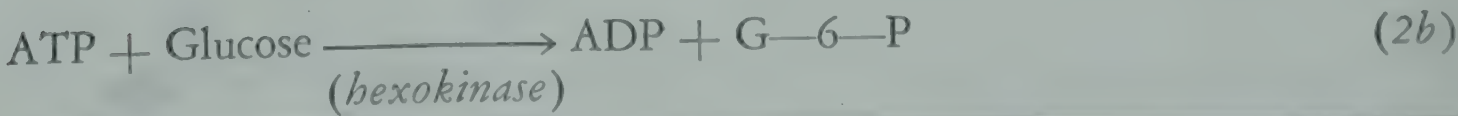
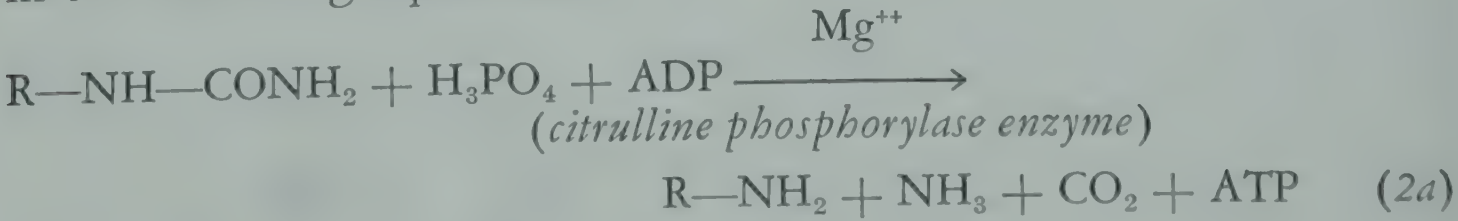
\* Buffered with maleate or phthalate.

From Korzenovsky and Werkman (10).

in concentration of orthophosphate, will appear greater in the presence of the hexokinase system. These observations also indicate that ATP is in fact the acid-labile phosphate compound which is formed as a result of citrulline degradation (10). Knivett (8) and Slade (21) have reached similar conclusions.

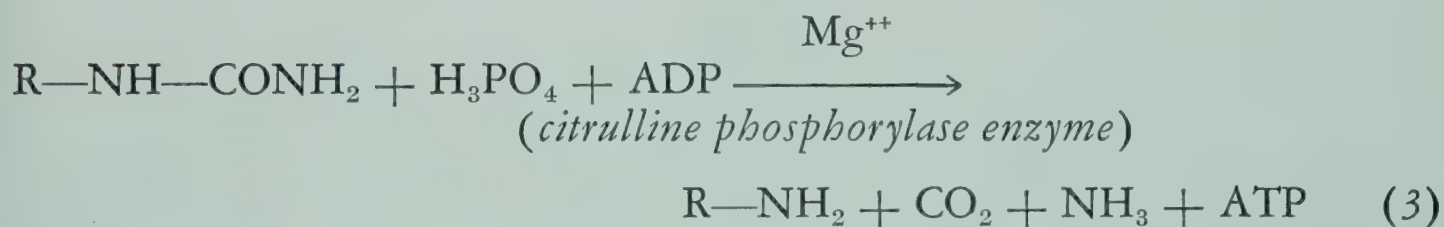
Because of the phosphorolytic nature of citrulline degradation it has been proposed that the conversion be designated as the citrulline phosphorylase reaction.

The stoichiometry has been established by coupling the citrulline phosphorylase reaction with the glucose hexokinase system as shown in the following equations:



The products determined are given as the sum of these two equations. Whether ATP, formed in the first reaction, is satisfactorily determined in terms of glucose-6-phosphate depends upon the absence or non-functioning of competing enzymes. Although crude extracts catalyze the release of orthophosphate from ATP, a marked inhibition occurs in the presence of hexokinase and glucose (Table 6). Even greater inhibition is achieved when ATP is added in  $0.1 \mu M$  aliquots. Orthophosphate is also released from ADP. In the presence of hexokinase and glucose this does not occur, but the concentration of glucose decreases (11). It thus appears that ADP is not destroyed hydrolytically but that the extracts contain adenylate kinase. It has been concluded that in the presence of the hexokinase system these two pathways of ATP destruction are inhibited to negligible levels. The ATP formed in the citrulline phosphorylase reaction is almost quantitatively determined in terms of glucose-6-phosphate.

A complete chemical balance appears in Table 7. The data show that the reaction proceeds in accordance with the following equation:



Data on the reversibility of the citrulline phosphorylase reaction are given in Table 8. Fixation of  $\text{C}^{14}\text{O}_2$  occurs both in exchange-type experiments and those intended to show a net synthesis of citrulline. The indispensability of ATP for fixation of  $\text{C}^{14}\text{O}_2$  into citrulline by exchange has been shown by addition of hexokinase and glucose to the test system. As would be expected, ATP is also necessary for synthesis of citrulline. Fluoride is a potent inhibitor of both degradation and synthesis. No involvement of carbamyl-L-glutamic acid or acetyl-L-glutamic acid could be shown with either dialyzed or undialyzed extracts. It is evident from the data of Table 9 that the radioactivity of citrulline is due solely to  $\text{C}^{14}$  present in the carbamyl group.



TABLE 6

RELEASE OF ORTHOPHOSPHATE FROM ADENOSINE DIPHOSPHATE AND  
ADENOSINE TRIPHOSPHATE

Indicated additions were made to a test system which contained 0.1 ml. extract (4.5 mg. protein), 15  $\mu$ M. orthophosphate, 0.04 M. maleate buffer, pH 6.2, 20  $\mu$ M.  $MgCl_2$ , and 10  $\mu$ M. iodoacetate. Total volume 1.1 ml.; incubated 30 minutes. Protein was precipitated with ammonium sulfate saturated in 0.1 N acetate buffer, pH 4.0.

No.	Additions	$+\Delta \mu M. P$
1.	ATP, 5 $\mu$ M. ....	4.97
2.	ATP, 5 $\mu$ M., 56 K. M. units hexokinase, 50 $\mu$ M. glucose	0.75
3.	Same as No. 2, but 5 $\mu$ M. ATP aded in 0.1 $\mu$ M. aliquots	0.21
4.	ADP, 5 $\mu$ M. ....	0.89
5.	ADP, 5 $\mu$ M., 56 K. M. units hexokinase, 50 $\mu$ M. glucose	0.00

From Korzenovsky and Werkman (11).

TABLE 7

STOICHIOMETRY OF THE CITRULLINE PHOSPHORYLASE REACTION

The test system contained 0.1 ml. extract (4.3 mg. protein), 15  $\mu$ M. orthophosphate, 0.04 M. maleate buffer, pH 6.2, 20  $\mu$ M.  $MgCl_2$ , 10  $\mu$ M. iodoacetate, 0.2  $\mu$ M. adenosine, diphosphate, 25  $\mu$ M. L-citrulline, 50  $\mu$ M. glucose and 56 K. M. units hexokinase. Total volume 1.2 ml.; incubated 30 minutes.

Compound	$\Delta \mu M.$
Citrulline .....	—3.9
Orthophosphate .....	—3.7
Glucose .....	—3.5
Ornithine .....	+3.8
Carbon dioxide .....	+3.6
Ammonia .....	+3.8
Glucose-6-phosphate .....	+3.6

From Korzenovsky and Werkman (11).

TABLE 8

## REVERSIBILITY OF CITRULLINE PHOSPHORYLASE REACTION

The reaction mixture consisted of 0.2 M. phosphate buffer, pH 6.8-6.9, 50  $\mu$ M.  $\text{NH}_4\text{Cl}$ , 50  $\mu$ M.  $\text{MgSO}_4$ , 50  $\mu$ M. ATP,  $\text{NaHC}^{14}\text{O}_3$ , extract and substrate in a total volume of 5.5 ml.; gas, air. Twenty-five  $\mu$ M. of substrate were present in Nos. 1 and 2; 100  $\mu$ M. in Nos. 3 and 4. Undialyzed extract containing 9.9 mg. protein was used in Nos. 1 and 2. No. 3 contained another sample of undialyzed extract with 9.1 mg. protein. No. 4 contained the same sample of extract used in No. 3 but was dialyzed against 0.05 M. phosphate buffer, pH 6.2-6.3, for 6 hrs. The reaction mixture was incubated for 70 minutes at 30.4° C. Counts were made on aliquots of protein-free filtrate.

No.	Substrate	Additions	$\text{NaHC}^{14}\text{O}_3$ CPM	Total $\text{C}^{14}$ Fixed CPM
1.	Citrulline	None .....	707,872	3308
	Citrulline	CLG, 50 $\mu$ M. ....	707,872	3540
2.	Citrulline	None * .....	1,380,000	6980
	Citrulline	ATP replaced by ADP * .....	1,380,000	4640
	Citrulline	ATP replaced by ADP, 112 K.M. units hexokinase; 50 $\mu$ M. glucose .....	1,380,000	908
	None	None * .....	1,380,000	848
	Ornithine	None .....	1,380,000	2432
	Ornithine	$\text{NaF}$ , 50 $\mu$ M. ....	1,380,000	368
3.	Citrulline	None .....	1,111,600	10,330
	Citrulline	None .....	1,111,600	10,256
	Citrulline	CLG, 50 $\mu$ M. ....	1,111,600	10,042
	Ornithine	None .....	1,111,600	3094
	Ornithine	Without ATP .....	1,111,600	138
	None	None .....	1,111,600	2112
4.	Citrulline	None .....	1,000,200	8846
	Citrulline	CLG, 50 $\mu$ M. ....	1,000,200	8414
	Ornithine	None .....	1,000,200	912
	Ornithine	CLG, 50 $\mu$ M. ....	1,000,200	1002
	Ornithine	Without ATP .....	1,000,200	116
	None	None .....	1,000,200	208

\* With 50  $\mu$ M. iodoacetate.

TABLE 9

POSITION OF  $\text{C}^{14}$  FIXED IN CITRULLINE

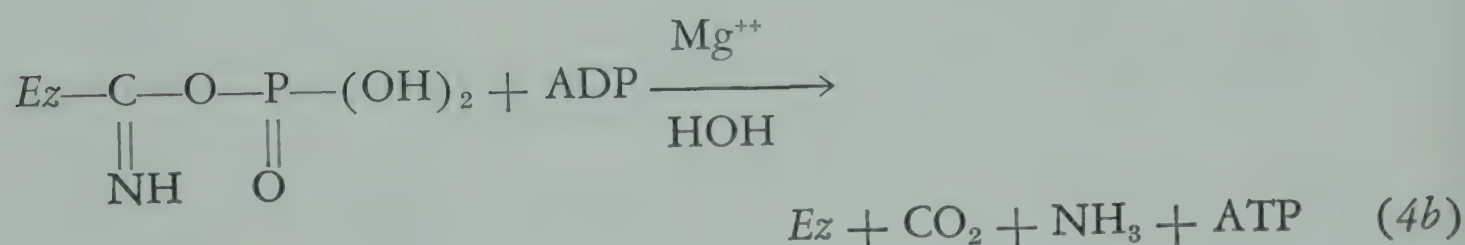
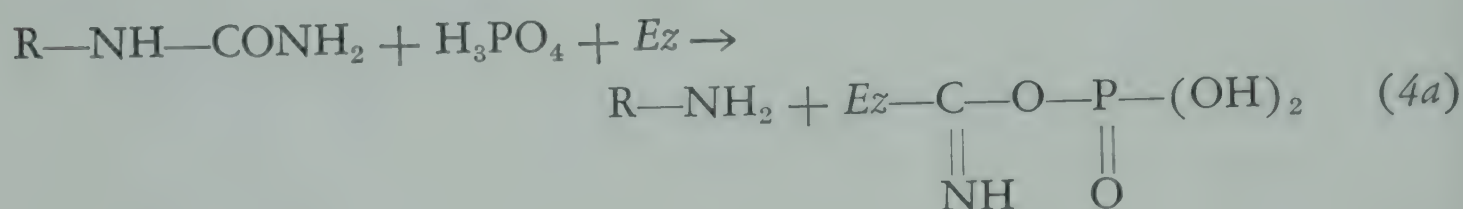
Experimental conditions as described in Table 8. Citrulline was isolated and purified by chromatography. Degradation was accomplished enzymatically to obtain the carb-amido carbon.

	CPM per $\mu$ M. of compound
Citrulline .....	441
$\text{CO}_2$ originating from carbamyl group	442



## MECHANISM OF CITRULLINE PHOSPHORYLASE REACTION

In a time-course study of the citrulline phosphorylase reaction it was found that the ratio  $\text{NH}_3$  (or  $\text{CO}_2$ ): P is nearly one and remains essentially constant with time. This is interpreted to indicate that the carbamyl group ( $\text{NH}_2\text{-CO-}$ ) of citrulline is split-off by a phosphoclastic type of reaction. The sequence of events may be depicted as follows:

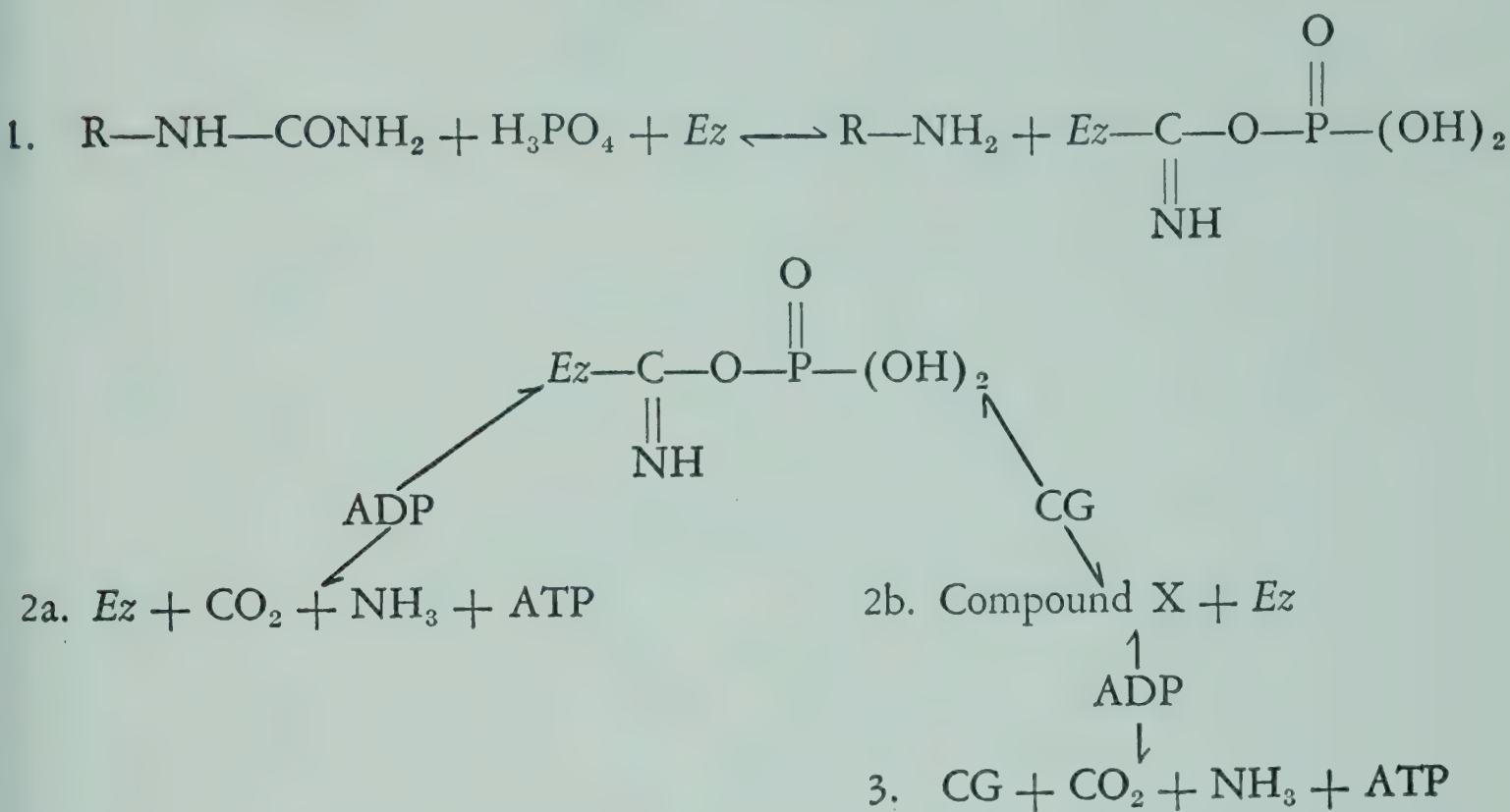


Reaction 4a represents the phosphoclastic split of citrulline that gives ornithine and a phosphorylated carbamic acid. Reaction 4b represents the transfer of high-energy phosphate to ADP and the decomposition of carbamic acid. Only reaction 4a is operative in the presence of arsenate because of the supposed instability of arsenyl carbamic acid. Ornithine and fluoride are inhibitory in the presence of phosphate or arsenate (10). The site of this inhibition is reaction 4a according to the proposed mechanism.

It has been shown that the fixation of  $\text{CO}_2$  and ammonia into citrulline requires high-energy phosphate which is supplied by ATP. The first step in the fixation may be reaction 4b. On the basis of the proposed mechanism, citrulline synthesis is favored by the removal of ADP, formed in reaction 4b. Synthesis is also favored by the absence of orthophosphate. A steady supply of ATP and removal of ADP could be accomplished by incorporation of the proper enzymes into the reaction mixture.

It is of interest to compare the proposed mechanism for the bacterial degradation and synthesis of citrulline with the better established mechanism for citrulline synthesis by mammalian liver.

This has been done in Fig. 1. Inasmuch as the nature of the phosphorylated intermediate is unknown, reaction 1 postulated for the bacterial system, is assumed to occur also in the liver enzymes.



### BACTERIAL ENZYMES

### LIVER ENZYMES

FIG. 1. Metabolism of citrulline by bacterial and liver enzymes.

After the formation of the phosphorylated carbamic acid by reaction 1 the pathways diverge in the two systems. In bacteria the phosphorylated carbamic acid is decomposed as previously described and yields the products given in reaction 2a. In liver the phosphorylated carbamic acid reacts with carbamyl-L-glutamic acid or a compound of similar nature (the identity of the naturally occurring substance has not been established), to form the Compound X of Grisolia and Cohen (reaction 2b). Compound X is further metabolized as indicated in reaction 3. It may be seen that the principal difference between the bacterial and liver systems is the involvement of carbamyl-L-glutamic acid in the latter. The function of carbamyl-L-glutamic acid appears to be that of a carrier of CO<sub>2</sub> and ammonia. The citrulline-synthesizing activity of the bacterial system is slight. On the other hand, the liver enzymes synthesize citrulline rapidly. This difference may be caused by the absence of an efficient CO<sub>2</sub> and ammonia carrier system in bacteria.



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# THE METABOLISM OF CITRULLINE BY BACTERIA \*

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I SHALL TRY here to summarize our current knowledge of the degradation of citrulline by bacteria, under the following headings: requirements of the enzyme system, the phosphate acceptor, formation of ATP, effect of arsenate, and general discussion.

In 1951 it was shown that heavy suspensions of *Streptococcus faecalis* cells released  $\text{NH}_3$  from citrulline upon prolonged incubation (1). It was proposed that citrulline was converted to ornithine,  $\text{CO}_2$ , and  $\text{NH}_3$ . Previous to this work, Hills (2) had shown that *Streptococcus* and *Staphylococcus* dissimilated arginine to ornithine,  $\text{CO}_2$ , and  $2\text{NH}_3$ . Citrulline was not attacked by whole cells, and it was consequently not believed to be an intermediate. It was also shown (2) that urease was absent in these organisms. The breakdown of arginine thus occurred by a reaction mechanism which differed from that of the arginase of mammalian liver.

It was clearly established in 1952 that citrulline was converted by *Streptococcus* to equimolar quantities of ornithine,  $\text{CO}_2$  and  $\text{NH}_3$  by cell-free extracts (8, 17), cells treated with cetyl-trimethyl-ammonium bromide (3), and acetone-dried cells (10), and by whole cells of *Clostridium* (12). The overall change was termed the citrulline ureidase reaction (13).

## REQUIREMENTS OF THE ENZYME SYSTEM

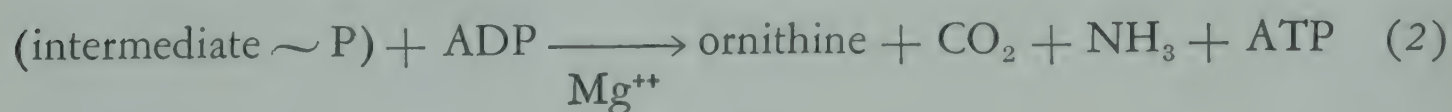
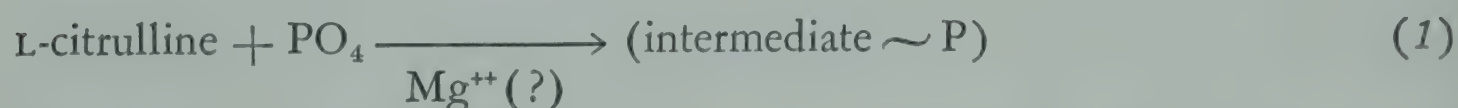
Simultaneous studies in several of these laboratories established in 1952 that acetone-dried cells of *S. faecalis* metabolized citrulline only upon the addition of ADP or larger quantities of ATP (4).

\* The investigations reported herein were supported by contract N7-onr-45002 between the Office of Naval Research and Northwestern University.



The complete requirements of the reaction with cell-free extracts were shown for the first time to be (a) inorganic phosphate, (b) AMP-5 or ADP, and (c) divalent ions (13, 17). Similar results were subsequently reported from other laboratories (9, 11). The enzyme system does not metabolize D-citrulline. Neither D-citrulline nor L-ornithine have been found in this laboratory to be inhibitory when tested with either arsenate, or phosphate plus ADP. D-Citrulline was reported to be inhibitory to the phosphate system in extracts of *S. lactis* (9). No enzymatic activity remained after holding the enzyme at 65° C. for 4 mins. and testing in the presence of phosphate plus ADP.

To facilitate the discussion which is to follow, the following limited scheme (14) is presented of the overall reaction. This scheme is not intended to represent the actual mechanism of the reactions involved.



#### PHOSPHATE ACCEPTOR

Adenosine monophosphate may be a primary phosphate acceptor in the citrulline ureidase reaction. Results have been obtained with acetone-dried cells (11) and cell-free extracts prepared by sonic oscillation (13, 15) or by shaking with glass beads in a Mickle disintegrator. Fig. 1 illustrates the activity of dialyzed cell-free extracts with equimolar quantities of AMP and ADP. *S. faecalis* (strain D10) was used. Myokinase and ATPase were present in these preparations. The activity of the latter enzyme in the sonic preparations was equivalent to the removal of about 10  $\mu$ moles P/hr./mg. protein. Practically no ADPase activity was present. Pyrophosphatase activity was also present in D10 sonic extracts. Consequently, apparent ATPase activity may be due to a liberation of pyrophosphate from ATP and a splitting of the latter to orthophosphate.

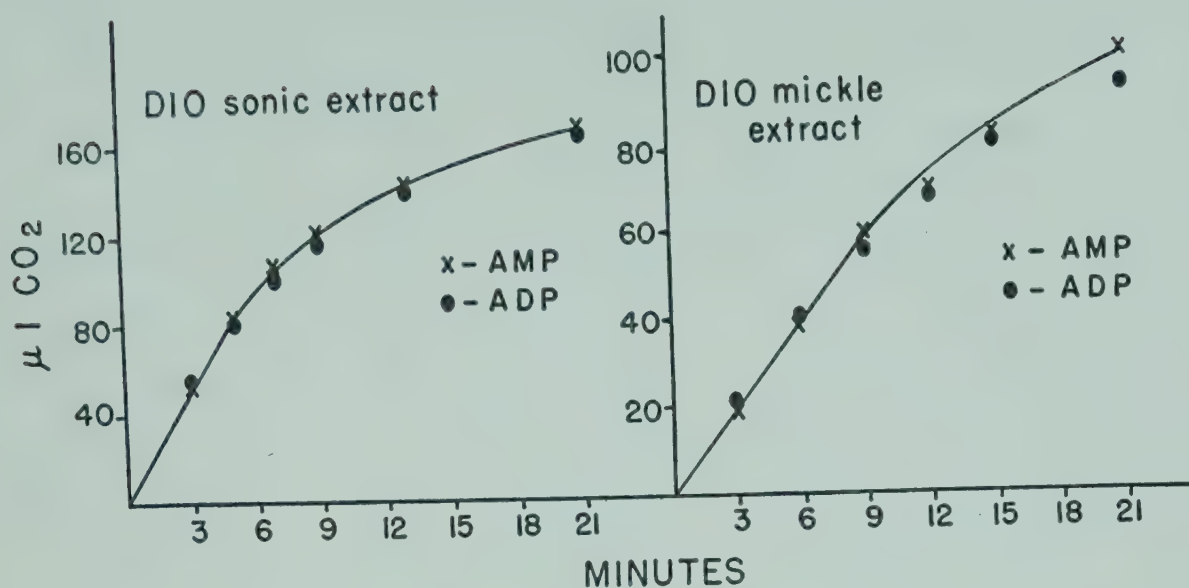


FIG. 1. The comparative activity of *Streptococcus* cell-free extracts prepared by sonic oscillation or Mickle disintegration. Cup contents: 9  $\mu\text{moles}$  L-citrulline, 2.9  $\mu\text{moles}$  AMP-5 or ADP, 10  $\mu\text{moles}$   $\text{Mg}^{++}$ , 250  $\mu\text{moles}$  phosphate pH 5.8. Sonic extract, 7.0 mg. protein; Mickle extract, 2.3 mg. protein. Total vol., 3 ml.

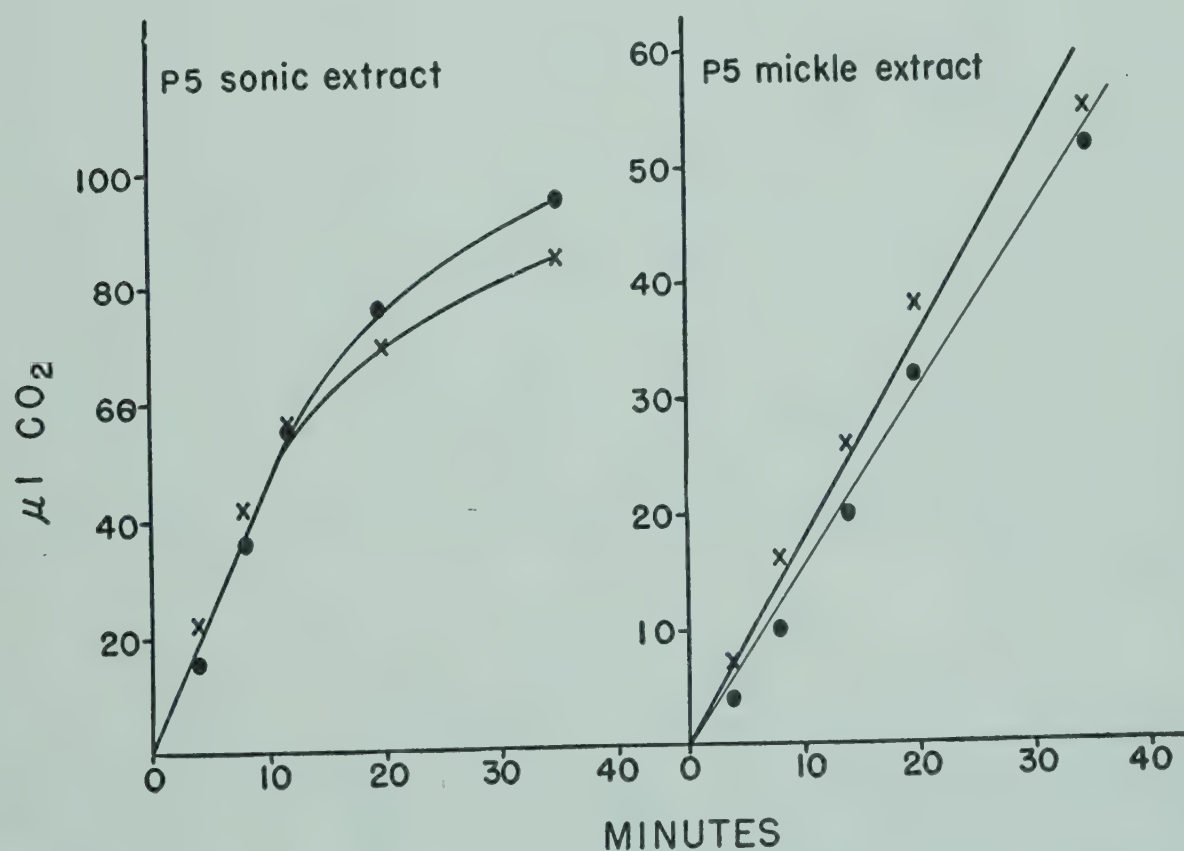


FIG. 2. The comparative activity of *Pseudomonas* cell-free extracts prepared by sonic oscillation or Mickle disintegration. Cup contents: 6  $\mu\text{moles}$  L-citrulline, 2.9  $\mu\text{moles}$  AMP-5 or ADP, 10  $\mu\text{moles}$   $\text{Mg}^{++}$ , 250  $\mu\text{moles}$  phosphate, pH 5.8. Mickle extract, 2.3 mg. protein; sonic extract, 5.4 mg. protein. Total vol., 3 ml.



The equivalent activity of AMP and ADP in these experiments may be related to the activity of ATPase and myokinase. In extracts of *Pseudomonas*, however, myokinase was of low activity and ATPase activity was one-sixth of that present in D10 extracts (15). Fig. 2 illustrates that AMP and ADP also possessed equivalent activity in this case. It seems likely that inability of AMP to function as primary phosphate acceptor would at least be evidenced by the presence of a lag period before maximum activity was attained. No such result has been obtained with either of these two organisms. That phosphate turnover did occur, however, is illustrated by the fact that a complete conversion of citrulline took place even in the presence of less than equivalent quantities of either phosphate acceptor. On the other hand, extracts of another strain of *S. faecalis* exhibited a lag period with AMP before maximum activity was attained. Also, the molar quantity of  $\text{CO}_2$  evolved was equal to the moles of ADP added and to twice the moles of AMP added (5). These results were interpreted to show that ADP acted as primary phosphate acceptor. The final answer must await the availability of enzyme preparations which do not possess enzymes responsible for phosphate turnover among the adenosine phosphates.

The effective removal of ATPase from streptococcal extracts by the usual fractionation procedures has not been completely satisfactory. In addition, treatment with Amberlite 1R-100 and Dowex-1 resins, or the use of heat, were not satisfactory. The enzyme was not inhibited by azide, arsenite, iodoacetate, cyanide, 2, 4-dinitrophenol, or  $\alpha, \alpha$ -dipyridyl.

#### FORMATION OF ATP

ATP is formed in the citrulline ureidase reaction in the presence of either AMP or ADP as phosphate acceptor. The compound was measured by a specific enzymatic assay (14, 15), separation by paper chromatography and chemical determination (6), and 7 minute hydrolysis in  $N$  HCl at  $100^\circ \text{C}$ . (11).

There seems little doubt that approximately 1 mole of inorganic phosphate is esterified for each mole of citrulline decomposed. The data in Table 1 show that with either AMP or ADP as phosphate

TABLE 1

FORMATION OF ATP FROM AMP-5 OR ADP IN PRESENCE OF EXCESS INORGANIC PHOSPHATE.

Cup contents: 0.5 ml. purified D10 enzyme containing 170  $\mu$ g. nitrogen; 6  $\mu$ moles L-citrulline, 250  $\mu$ moles phosphate, pH 5.8; 10  $\mu$ moles  $MgSO_4$ ; AMP-5 or ADP as indicated below; water to 3 ml. 37° C. Manometers were removed from water bath as the reaction rate slowed down (between 15 and 50 min.), contents placed in boiling water 8 min., cooled, and centrifuged.

The AMP contained 1.2% ATP and no ADP; the ADP contained 0.7% ATP.

P/CO<sub>2</sub> ratio calculated on basis of phosphorus required to form the ATP present and the carbon dioxide evolved (corrected for endogenous).

	$\mu$ moles AMP-5 added						$\mu$ moles ADP added					
	0	0.5	1.0	2.0	5.0	10.0	0	0.5	1.0	2.0	5.0	10.0
$\mu$ moles CO <sub>2</sub> evolved, first 8 min.	0.3	1.0	1.6	2.5	2.3	2.4	0.3	1.3	1.5	2.2	2.6	2.7
total $\mu$ moles CO <sub>2</sub> evolved	0.4	1.8	3.0	4.8	5.7	6.1	0.4	1.5	2.1	4.1	5.7	6.2
total $\mu$ moles CO <sub>2</sub> (corrected)	—	1.4	2.6	4.4	5.3	5.7	—	1.1	1.7	3.7	5.3	5.8
$\mu$ moles ATP formed	—	0.3	0.7	1.4	1.8	1.0	—	0.4	0.9	1.5	3.6	4.0
P/CO <sub>2</sub> ratio	—	0.43	0.54	0.64	0.68	0.35	—	0.36	0.53	0.41	0.68	0.69



acceptor a net synthesis of ATP occurred. This reached a value of about 0.7 mole of phosphate esterified for each mole of citrulline decomposed in spite of phosphate turnover. The larger quantities of AMP and ADP, as used in Table 1, favored ATP synthesis. This is likely due to changes in equilibrium conditions in each case. The data of Table 1 were obtained with a single purified enzyme preparation.

The purification process which was used is as follows: To 30 ml. dialyzed sonic extract was added 42 ml. (1.6 mg. dry wt.) calcium phosphate gel, and the pH was adjusted to 7.3 with dilute  $\text{NH}_4\text{OH}$ . The solution was stirred for  $\frac{1}{2}$  hr. and centrifuged for 10 mins. at 10,000 g. Twenty-five ml. (350 mg. dry wt.) alumina C $\gamma$  was added, and the pH adjusted to 6.8 with dilute acetic acid. The solution was stirred  $\frac{1}{2}$  hr. and centrifuged. The precipitate was then eluted with 9, 6, 6, and 6 ml. aliquots of 0.05 M. phosphate, pH 7.5. The eluates were combined and treated with 10 ml. (380 mg. dry wt.) calcium phosphate gel and the supernatant with 10 ml. (140 mg. dry wt.) alumina C $\gamma$ . The alumina precipitate was then eluted as above, and the eluate dialyzed over-night. The entire process was carried out at 5° C. The activity of the various fractions is given in Table 2. The specific activity of the phosphorolytic system was increased 10 times in this preparation, while the ATPase activity remained constant.

TABLE 2

PURIFICATION OF CITRULLINE UREIDASE ENZYME SYSTEM IN *Streptococcus* EXTRACTS

Step	Mg. protein per ml.	Mg. protein total	Specific activity	Total activity	Per cent original activity
Original	19.4	592	3.0	1776	—
PO <sub>4</sub> gel supernate	4.9	309	5.7	1760	99
Alumina C $\gamma$ eluate	2.8	92	12.2	1128	64
PO <sub>4</sub> gel supernate	1.8	47	22.0	1035	59
Alumina C $\gamma$ eluate	1.3	34	28.1	950	53

All fractions were assayed with 6  $\mu$ moles L-citrulline, 250  $\mu$ moles phosphate, pH 5.8, 3  $\mu$ moles ADP, 10  $\mu$ moles  $\text{Mg}^{++}$ .

Other experiments with cell-free extracts demonstrated a net synthesis of 0.6 mole ATP from ADP per mole citrulline decomposed. The use of  $P^{32}$  showed clearly that phosphate turnover, as catalyzed by ATPase and myokinase, did occur (6). Acetone-dried cells of *S. faecalis*, in the presence of AMP, synthesized 1 mole of

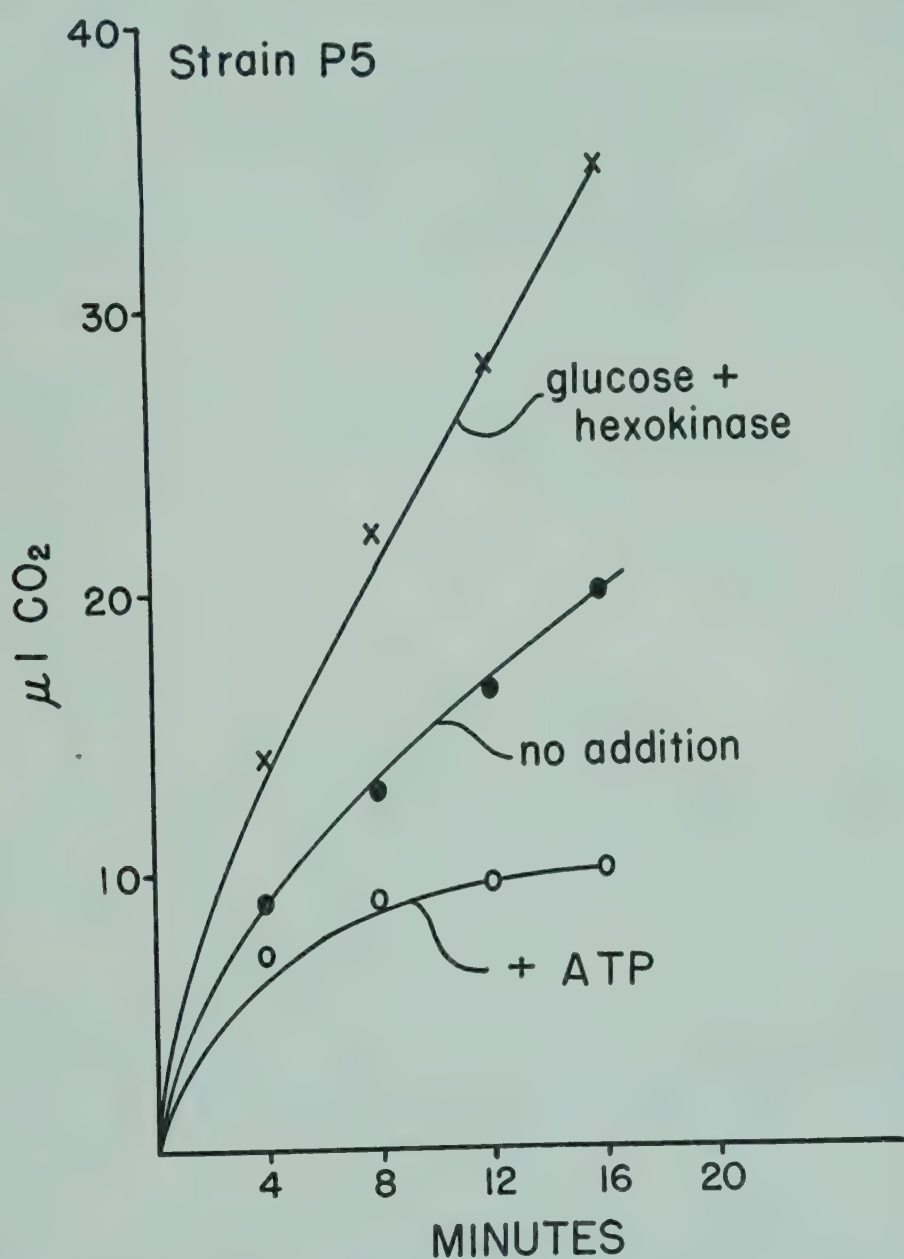


FIG. 3. The effect of hexokinase + glucose, and ATP on the rate of liberation of  $CO_2$  from citrulline by cell-free sonic extract of *Pseudomonas*. Cup contents: 6  $\mu$ moles L-citrulline, 5.5 mg. enzyme protein, 250  $\mu$ moles phosphate pH 5.8, 3.5  $\mu$ moles ADP, 10  $\mu$ moles  $Mg^{++}$ ; 7  $\mu$ moles ATP, or 20  $\mu$ moles glucose and 100  $\mu$ g. hexokinase (Pabst) were added where indicated. Total vol., 3 ml.

7 min. acid-hydrolyzable phosphate per mole of citrulline decomposed (11).

Dialyzed extracts of *Pseudomonas* contain about one-sixth the ATPase activity of *Streptococcus* extracts (15). This weak activity is reflected in an inhibitory effect of ATP on the rate of citrulline



breakdown. In the presence of glucose and hexokinase the reaction rate was greater than the control. A typical set of results is illustrated in Fig. 3. In unpurified extracts of *Streptococcus* such effects are not as marked.

It has been found that neither carbamyl-L-glutamate ( $16.6 \times 10^{-3} M.$ ) nor carbamyl-DL-aspartate ( $33.2 \times 10^{-3} M.$ ) were inhibitory to the metabolism of citrulline by extracts of *S. faecalis* in the presence of phosphate and ADP. The same result was obtained in the presence of arsenate. Thus, if more than one enzyme is involved in the degradation of citrulline, they were not inhibited by the above compounds.

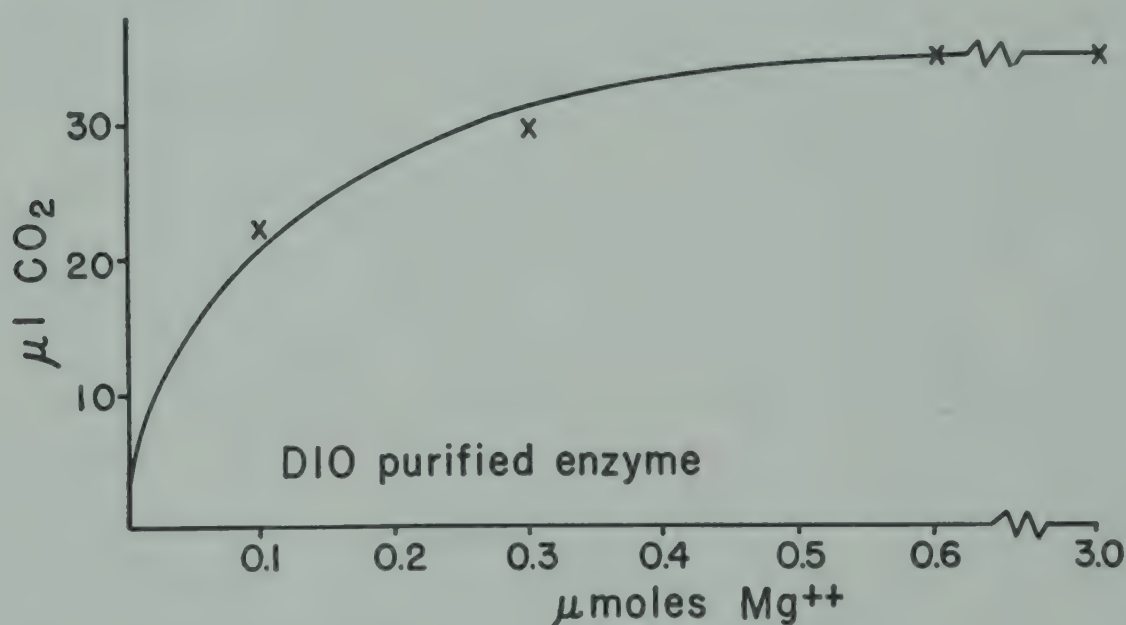


FIG. 4. The effect of  $Mg^{++}$  on the release of  $CO_2$  from citrulline by a purified enzyme from *Streptococcus*. Cup contents: 6  $\mu$ moles L-citrulline, 250  $\mu$ moles phosphate pH 5.8, 5.4  $\mu$ moles ADP, 0.5 mg. enzyme protein;  $MgSO_4$  added as indicated. Total vol., 3 ml.

Fig. 4 illustrates the requirement for  $Mg^{++}$  by a purified enzyme when tested with phosphate and ADP. The absence of any effect when  $Mg^{++}$  was added to the arsenate system, in the same preparation, demonstrates that an additional reaction occurred in the phosphate system which required  $Mg^{++}$ .

#### EFFECT OF ARSENATE

*Formation of arsenyl intermediate.* Arsenate was shown to replace phosphate in the citrulline ureidase reaction in cell-free extracts of *S. faecalis* and *Pseudomonas* (13, 15). Under such conditions

phosphate acceptor and divalent ions were not required (9). The products formed from citrulline were the same as in the presence of phosphate. The ability of arsenate to replace phosphate, phosphate acceptor, and divalent ions is in agreement with known examples in which arsenate is able to form an arsenyl intermediate in place of the phosphoryl intermediate. Such an arsenyl compound

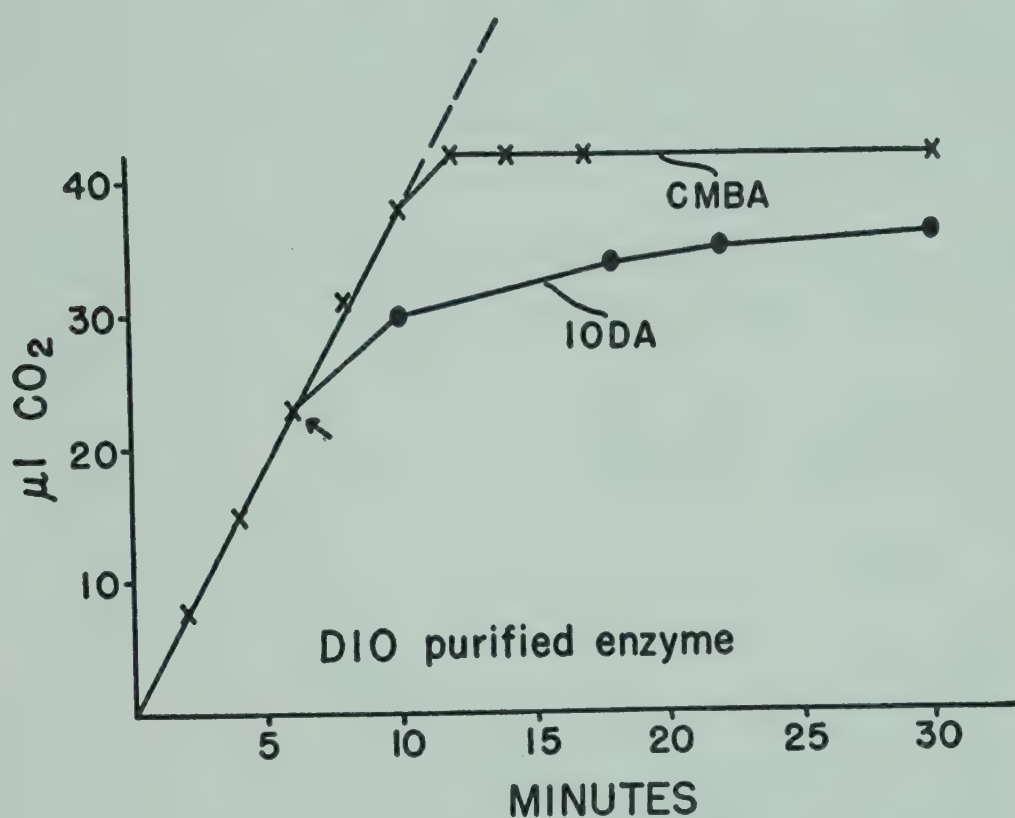
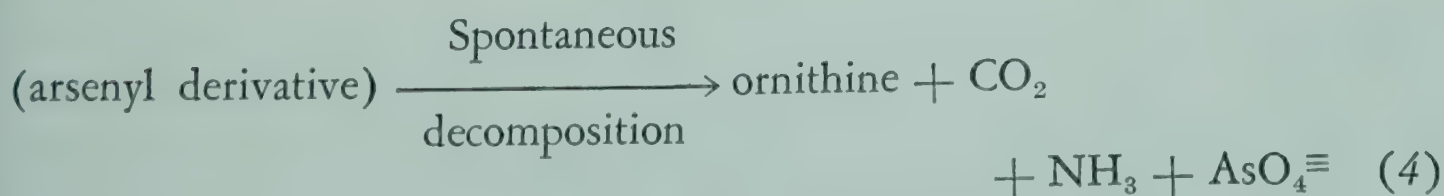
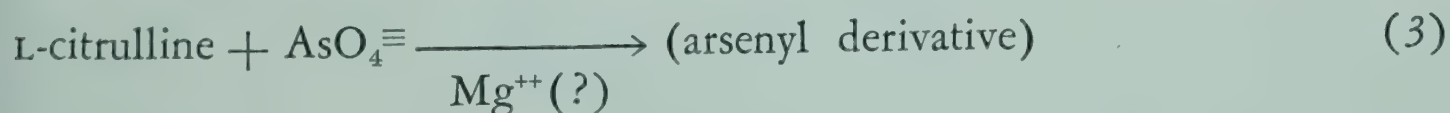


FIG. 5. The effect of iodoacetic acid (IODA) and *p*-chloromercuribenzoic acid (CMBA) on the arsenolysis of citrulline by purified enzyme from *Streptococcus*. Cup contents: 6  $\mu$ moles L-citrulline, 250  $\mu$ moles acetate pH 5.8, 30  $\mu$ moles  $\text{KH}_2\text{AsO}_4$ , 0.5 mg. enzyme protein; 5  $\mu$ moles IODA or CMBA added at time indicated. Total vol., 3 ml.

would decompose spontaneously, and result, in the case of citrulline, in the formation of equimolar quantities of ornithine,  $\text{CO}_2$  and  $\text{NH}_3$ . Such a scheme has been proposed (15) and is illustrated by the following reactions:





This scheme is also not intended to represent the actual mechanism of the reactions involved. Several other proposals of similar nature have been made (7, 11). In agreement with the above scheme it has been shown that phosphate is a competitive inhibitor of arsenate (7, 15). The purified enzyme was also inhibited by iodoacetate and *p*-chloromercuribenzoate (Fig. 5), a fact indicating that an SH group is involved in the action of the enzyme. Under the conditions described in Fig. 5, 20  $\mu$ moles L-cysteine showed a 50 per cent reactivation of the inhibition by the mercury compound after a short lag period.

*Inhibition by fluoride.* Fluoride is an effective inhibitor of arsenolysis (7, 9, 11, 13). This inhibition occurred in the apparent absence

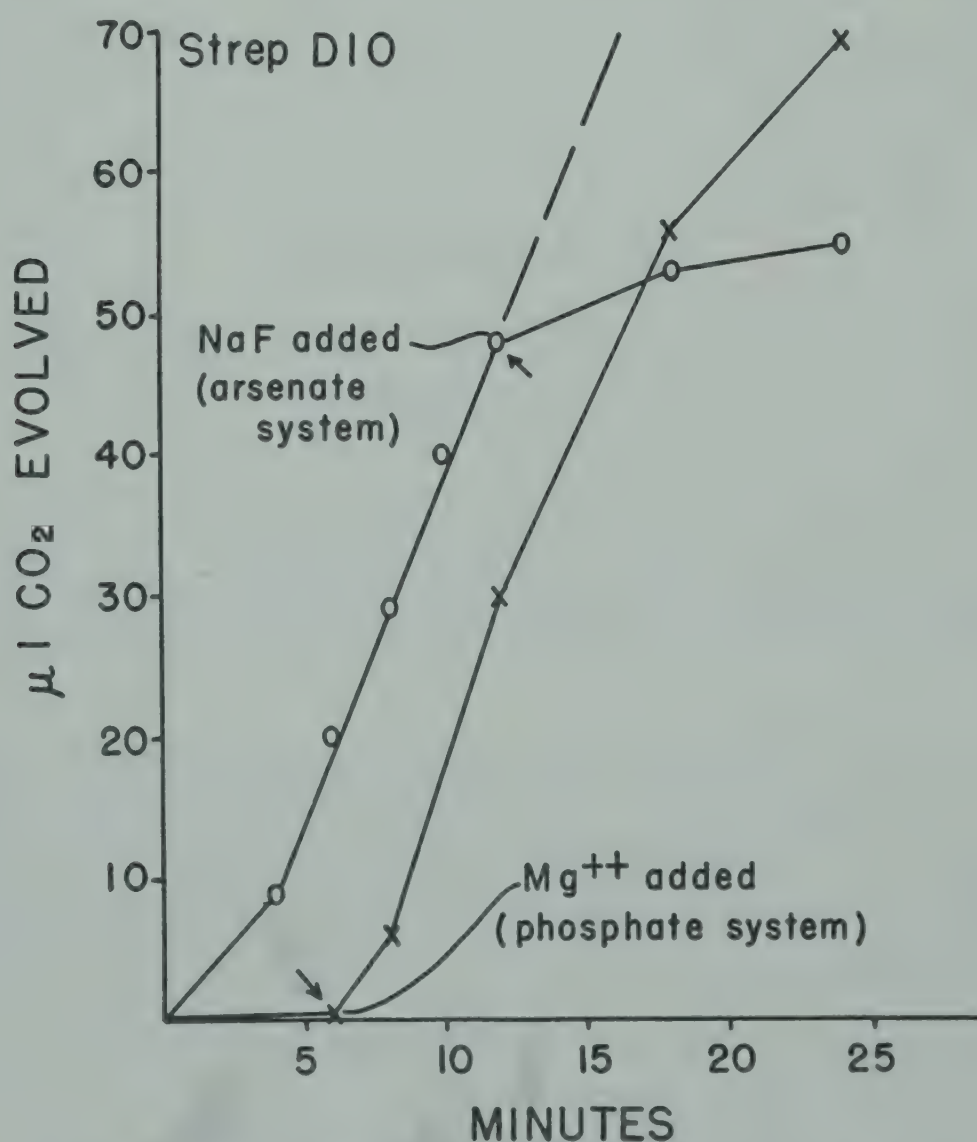


FIG. 6. The effect of fluoride in the arsenate system, and  $Mg^{++}$  in the phosphate system on the release of  $CO_2$  from citrulline by a purified enzyme from *Streptococcus*. Cup contents: phosphate system, 250  $\mu$ moles phosphate pH 5.8, 3  $\mu$ moles ADP and 20  $\mu$ moles  $MgSO_4$ ; arsenate system, 250  $\mu$ moles acetate pH 5.8, 15  $\mu$ moles NaF. Six  $\mu$ moles L-citrulline and 1.4 mg. enzyme protein were included in each case. Total vol., 3 ml.

of divalent ions. Fluoride is a common inhibitor of phosphorylation in many types of living cells and is believed to function by the formation of an inactive metallo-protein-enzyme complex. It thus seemed of interest to examine further the effect of fluoride on the arsenolysis of citrulline. Recent results (16) established first that the enzyme responsible for the formation of the arsenyl intermediate

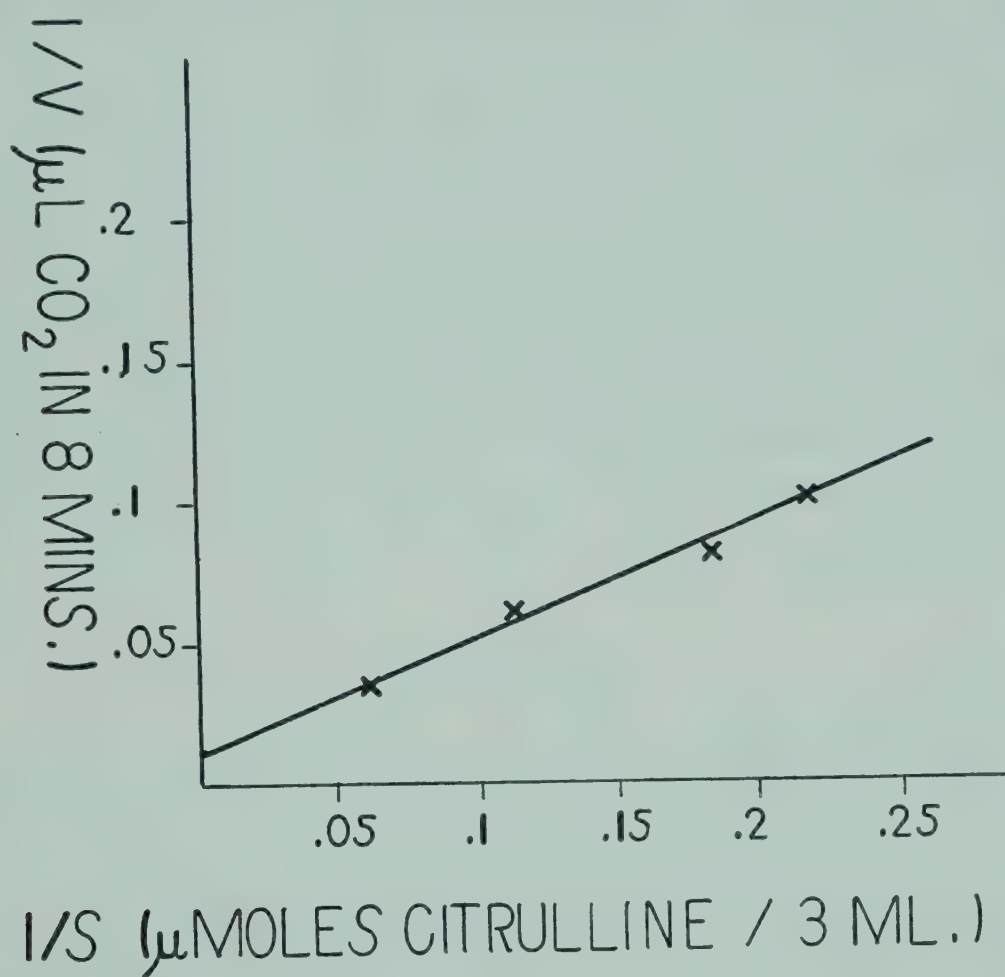


FIG. 7. The effect of L-citrulline on the evolution of  $\text{CO}_2$  by a cell-free extract of *S. faecalis*. Cup contents: 250  $\mu\text{moles}$  acetate pH 5.8, 30  $\mu\text{moles}$   $\text{KH}_2\text{AsO}_4$ , citrulline as indicated. Total vol., 3ml.

was identical with that responsible for the formation of phosphoryl intermediate. A divalent ion-free enzyme solution (as judged by assay in the phosphate system) was prepared. Fig. 6 illustrates the effect of  $\text{Mg}^{++}$  when added to the enzyme on the rate of decomposition of citrulline in the presence of  $\text{PO}_4^{=}$  and ADP. Arsenolysis catalyzed by the enzyme preparation was markedly inhibited by fluoride (Fig. 6), and its activity was not affected when  $\text{Mg}^{++}$  was added. Treatment of the enzyme by Versene did not alter its behavior to fluoride or  $\text{Mg}^{++}$  in the arsenolysis reaction.



It is thus apparent that if divalent ions are required for reaction (1) such ions are capable of complexing with fluoride but are incapable of reaction with Versene. These ions may be so situated in the protein matrix of the enzyme as to be inaccessible to Versene but yet be displaced by fluoride ions by virtue of size and affinity. The possibility of course exists that the inhibitory effect of fluoride is due to some action other than the binding or displacement of divalent ions (16).

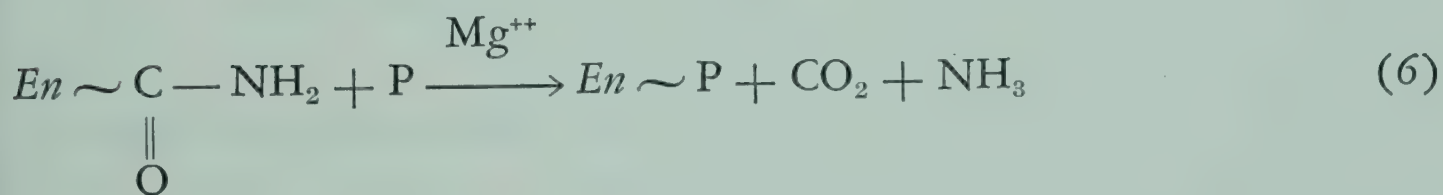
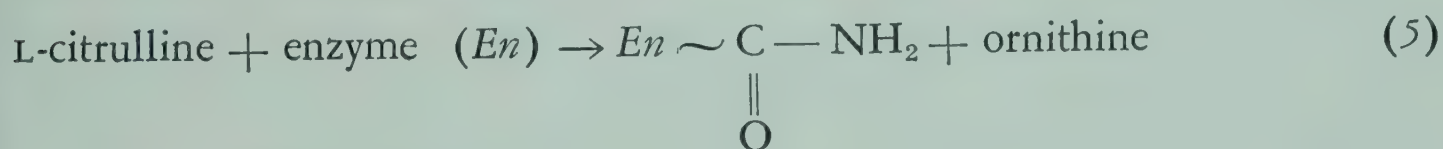
In order to determine the Michaelis constant of the arsenolysis of citrulline, the reciprocal of the velocity has been plotted against the reciprocal of the citrulline concentration (Fig. 7). The  $K_m$  value as calculated from these data is 0.10 M. This value is of the same order as that reported (7) for another strain of *S. faecalis*. This high  $K_m$  value explains the drop in rate of arsenolysis obtained when approximately one-half of the citrulline had decomposed (15).

### DISCUSSION

For the first time the breakdown of an amino acid (citrulline) has been shown to result in the esterification of inorganic phosphate in ATP. It has been known for some time that in the alcoholic fermentation of glucose to pyruvic acid there is a net gain of two energy-rich phosphate bonds per mole of glucose fermented. These reactions are concerned with triose-phosphate compounds originating in glucose. The present results demonstrate that citrulline (originating in arginine) may be an important source of energy for the growth of streptococci in nature. The additional requirement of arginine for protein synthesis would reduce the amount of the amino acid available to the cell as a source of energy. The lack of adequate carbohydrate, however, would increase the importance of "arginine-energy" for growth of the cell. Most streptococcal strains examined possessed significant quantities of the arginine dihydrolase enzyme system (2, 14). It is likely that this additional function of arginine, as compared to other amino acids, may be of considerable economic significance in the life of streptococci.

The nature of a phosphorylated intermediate which might be formed in the degradation of citrulline is of great interest. On a chemical basis, phosphate derivatives of citrulline could be written with a N—P linkage (as in arginine phosphate) or with a C—O—P linkage with the carbonyl carbon atom (11). The thermodynamic aspects of the enzymatic formation of these compounds make it unlikely that either one is formed in the bacterial system. Energy sufficient to account for the phosphorylation does not appear to be available. The cleavage of citrulline to ornithine and a "carbamyl unit," however, may liberate sufficient energy eventually to form a high-energy phosphate bond in ATP.

A mechanism of citrulline degradation based on the formation of a "carbamyl unit" may be pictured as follows. The reactions could occur on the enzyme surface and the high-energy bonds are assumed to involve the enzyme protein.



This scheme is, in general, consistent with the results presented in this report. According to reaction (6), the addition of  $\text{Mg}^{++}$  should result in the immediate release of  $\text{CO}_2$ . The results shown in Fig. 6 are in agreement. The release of  $\text{CO}_2$  and  $\text{NH}_3$  in equimolar quantities during the degradation of citrulline is also consistent with the scheme. In addition, the comparative activity of extracts during purification when tested with phosphate or arsenate had indicated that a single enzyme is concerned in the overall reaction (16). The competitive inhibition of arsenate by phosphate is likewise to be expected. The sulfhydryl nature of the enzyme indicates that an SH group may be concerned in the formation of the high energy bonds.



Contrary to the scheme, phosphate acceptor is required for the release of  $\text{CO}_2$  and  $\text{NH}_3$  from citrulline. It is likely that reactions (6) and (7) cannot be cleanly separated as visualized above but should be considered as interdependent. Also, it has not been possible with either crude or purified extracts of *Streptococcus* and *Pseudomonas* to demonstrate the presence of a compound which would react with hydroxylamine. The scheme is presented only as a hypothesis to be modified by further experimentation.

#### ACKNOWLEDGMENT

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# ON THE GLUTAMATE-PROLINE-ORNITHINE INTERRELATION IN VARIOUS MICROORGANISMS

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THE CONSIDERABLE biochemical attention which has been devoted to the amino acids glutamic acid, proline, and ornithine can probably be attributed not only to their many interesting features as individuals, but also to the early recognition of their possible metabolic connections. Thus, the central physiological role of glutamate, the participation of ornithine in the "ornithine cycle," and the ring structure of proline have no doubt contributed to the interest in these amino acids, as has the finding of their metabolic interrelation suggested by the pioneer nutritional studies of the beginning of this century. The historical development of this subject with special reference to animal studies is reviewed in this volume by Stetten, who has added so much knowledge to this field.

The present paper is restricted to the glutamate-proline-ornithine interrelation in *Escherichia coli*, *Neurospora crassa*, and *Torulopsis utilis*. These microorganisms offered from the experimental point of view certain desirable features which made it possible to study not only the existence of metabolic links involving these amino acids, but also the biosynthetic significance of such links.

## THE OVERALL METABOLIC CONNECTIONS INVOLVING GLUTAMATE, PROLINE, AND ORNITHINE IN MICROORGANISMS

In *E. coli*, glutamate has been shown to be a precursor of proline (1, 2, 3, 4, 5) and of ornithine (4, 6, 5); and a similar relationship appears to hold in fungi (7, 8, 9, 10, 11). Additional metabolic connections, not directly involving glutamate, between ornithine

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and proline have been found in *E. coli* (12, 13), *N. crassa* (14, 15, 10, 11), and *T. utilis* (11). Moreover, in all three of these organisms, glutamate, as well as proline and ornithine, have been shown to be closely related to glutamic  $\gamma$ -semialdehyde (3, 16, 15, 6, 9, 10, 11, 13). Details of the various interrelations will be presented below.

### GENERAL METHODS

In analyzing the relationships encountered, it was found necessary to apply a variety of methods. Some of the experimental approaches which proved particularly useful are listed below:

- (a) precursor accumulation by mutants, with or without "trapping" (3, 6);
- (b) growth response of mutants to accumulated or synthetic substances (1, 7, 2, 14, 3, 16, 17, 6, 10);
- (c) metabolic activity of cellular or mycelial pad suspensions of singly or multiply blocked mutants, with or without "trapping" (6, 9, 10);
- (d) selective inhibition of metabolic reactions (10);
- (e) demonstration of enzymes in extracts of wild-type organisms (17, 15, 6, 18);
- (f) demonstration of the absence of enzymes in extracts of mutants (17, 6); and
- (g) tracer incorporation and isotopic competition (8, 4, 12, 5, 11, 13).

### PROLINE PATHS IN *E. coli*, *N. crassa*, AND *T. utilis*

The major paths of proline synthesis in *E. coli*, *N. crassa*, and *T. utilis* have all been shown to involve the same sequence: glutamate, glutamic  $\gamma$ -semialdehyde,  $\Delta^1$ -pyrroline-5-carboxylate, proline (see Figs. 1 and 2).<sup>1</sup> In *E. coli*, this sequence was demonstrated

<sup>1</sup> The mechanism of the conversion of glutamate to glutamic  $\gamma$ -semialdehyde is unknown. It is possible that  $\gamma$ -glutamyl phosphate is an intermediate in this conversion, in analogy with the recent finding (19) that aspartate forms aspartic  $\beta$ -semialdehyde via  $\beta$ -aspartyl phosphate. In the two figures the arrows are given in the direction of biosynthesis; no implications are intended as to the possible reversibility of the steps involved.

through an analysis of mutant strains with respect to growth responses and precursor accumulation (3). Of three mutant strains investigated, one was found to give a growth response to proline, one to proline or glutamic  $\gamma$ -semialdehyde, and the third to proline,

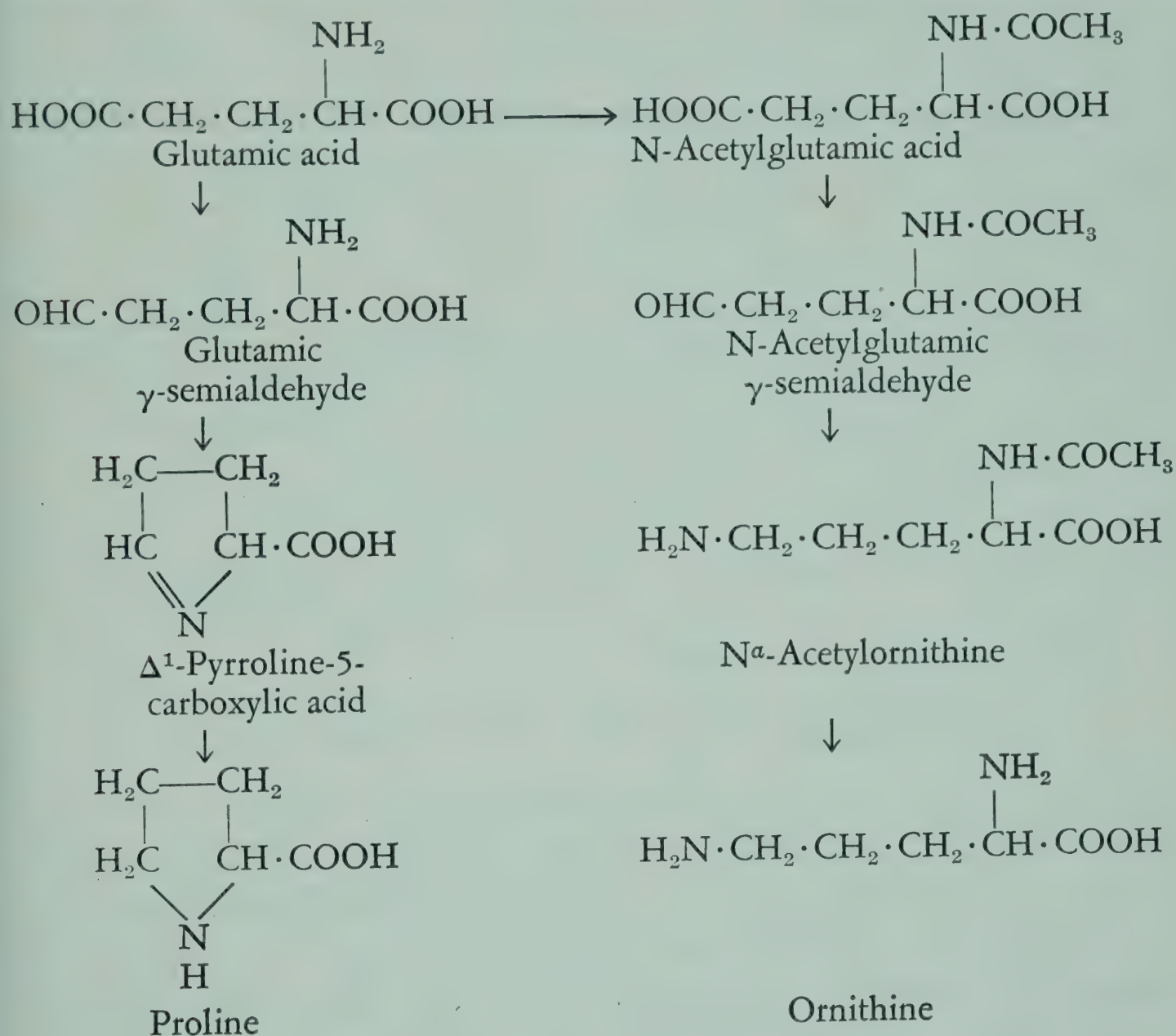


FIG. 1. Major paths of proline and ornithine synthesis in *Escherichia coli* (from Vogel, 6).

glutamic  $\gamma$ -semialdehyde, or glutamate. The mutant strain which responded only to proline excreted glutamic  $\gamma$ -semialdehyde into its culture medium (3). This study was facilitated by the development of a synthetic method for the preparation of glutamic  $\gamma$ -semialdehyde. The synthesis involved treatment of  $\gamma,\gamma$ -dicarbethoxy- $\gamma$ -actamidobutyraldehyde (20) with hot hydrochloric acid, so as to hydrolyze the ester and acetyl groups and decarboxylate to give glutamic  $\gamma$ -semialdehyde in almost quantitative yield (3). The semialdehyde



was found to be in equilibrium with its intramolecular cyclization product,  $\Delta^1$ -pyrroline-5-carboxylate; under physiological pH conditions the cyclized form appeared to predominate. It was also shown that the accumulation of glutamic  $\gamma$ -semialdehyde by the strain

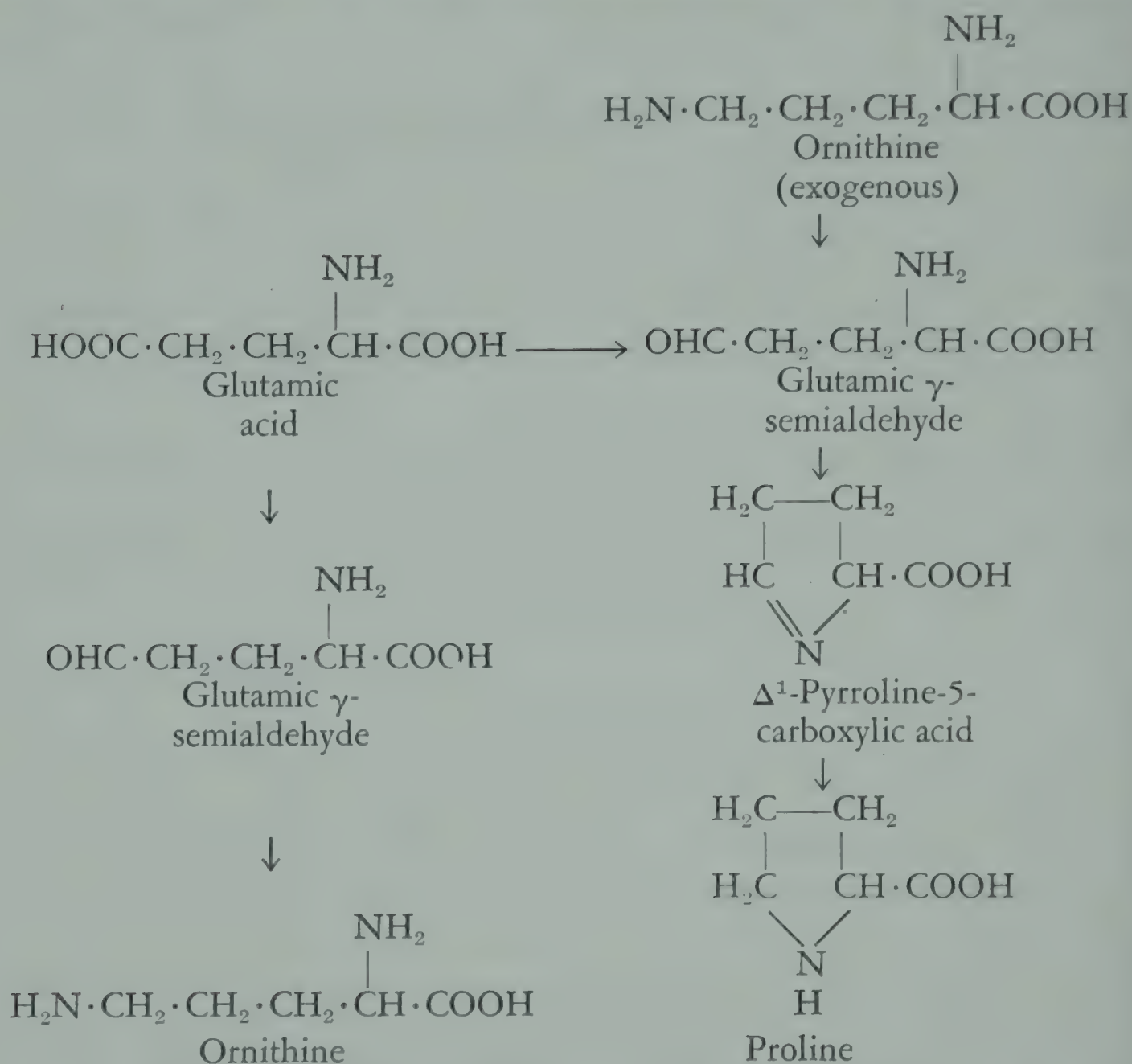


FIG. 2. Glutamate-proline-ornithine interrelation in *Neurospora crassa* and *Torulopsis utilis* (from Vogel and Bonner, 10; and Abelson and Vogel, 11). Glutamic  $\gamma$ -semialdehyde is shown twice to emphasize the possibility that the semialdehyde as proline precursor is distinct from the semialdehyde as ornithine precursor.

responding only to proline is considerably enhanced by the presence of *o*-aminobenzaldehyde, which appeared to function as a specific "trapping" agent, reacting with the glutamic  $\gamma$ -semialdehyde to form a dihydroquinazolinium compound. The latter compound is sufficiently stable to aid in accumulation, but sufficiently dissociable into its components to make the semialdehyde available as a growth

factor for the mutants which can utilize it (3). Consistent with these mutant studies were the results of isotopic competition experiments with wild-type *E. coli* (12, 5).

In *N. crassa*, the major proline path (see Figure 2) was also established by the use of mutants (10). One mutant is known which responds to proline but not to glutamic  $\gamma$ -semialdehyde, and another mutant was found to utilize glutamic  $\gamma$ -semialdehyde to satisfy a proline requirement. The mutant strain which cannot utilize glutamic  $\gamma$ -semialdehyde does not spontaneously accumulate this compound, as did the corresponding *E. coli* mutant; however, on incubation of mycelial pads of the *Neurospora* mutant with glutamate and *o*-aminobenzaldehyde as trapping agent, excretion of glutamic  $\gamma$ -semialdehyde could be demonstrated (10). Independent evidence for the major proline path in *N. crassa* was obtained with isotopic competition experiments (11). Analogous experiments with *T. utilis* showed that this organism synthesizes proline in the same manner as do *E. coli* and *N. crassa* (11).

#### ORNITHINE PATH IN *E. coli*

In *E. coli*, the major ornithine path was found to be represented by the following sequence: glutamate, N-acetylglutamate, N-acetylglutamic  $\gamma$ -semialdehyde, N <sup>$\alpha$</sup> -acetylornithine, ornithine (see Fig. 1). The evidence for this path also comes from an investigation of mutants (6) and is supported by results from enzyme (17, 6, 18) and tracer (12, 5) studies. N <sup>$\alpha$</sup> -acetyl-L-ornithine<sup>2</sup> has been isolated from culture filtrates of an ornithine-requiring mutant strain of *E. coli*<sup>3</sup> (17, 6), and has also been synthesized. The synthesis of this compound involved acetylation of N <sup>$\delta$</sup> -carbobenzoxy-L-ornithine (22) followed by hydrogenolysis of the resulting N <sup>$\alpha$</sup> -acetyl-N <sup>$\delta$</sup> -carbobenzoxy-L-ornithine to yield N <sup>$\alpha$</sup> -acetyl-L-ornithine (17). The latter compound satisfied the growth requirement of another ornithineless

<sup>2</sup> Melting point, 226-227° C.;  $[\alpha]_D^{25}$  6.8° (2% in water).

<sup>3</sup> Interestingly enough, N <sup>$\delta$</sup> -acetyl-L-ornithine also occurs in nature: it has been isolated from *Corydalis ochotensis* (21). No evidence could be found that the  $\delta$ -substituted compound (generously furnished by Dr. R. H. F. Manske) can function as a source of ornithine in *E. coli*.



mutant (17). The mutant which was found to excrete the  $N^{\alpha}$ -acetylornithine also accumulated in its culture filtrate  $N$ -acetylglutamic  $\gamma$ -semialdehyde (6). The role of  $N$ -acetylglutamate and  $N^{\alpha}$ -acetylornithine as ornithine precursors has also been demonstrated in tracer experiments (12).

Three of the enzymes participating in the *E. coli* ornithine path have been obtained in cell-free extract. A coenzyme-A-dependent enzyme has been found which converts glutamate to  $N$ -acetylglutamate (18). A pyridoxal-phosphate-stimulated enzyme, in the presence of glutamate, has been shown to catalyze the conversion of  $N$ -acetylglutamic  $\gamma$ -semialdehyde to  $N^{\alpha}$ -acetylornithine (6). The name acetylornithine  $\delta$ -transaminase might be appropriate for this enzyme.<sup>4</sup> The step from  $N^{\alpha}$ -acetylornithine to ornithine is mediated by a cobaltous-ion-stimulated enzyme (6), for which the name acetylornithinase has been proposed (23). No acetylornithinase activity could be detected in extracts of the mutant which accumulates  $N^{\alpha}$ -acetylornithine (17, 6). Acetylornithinase has also been studied from the point of view of its formation (24).

#### ORNITHINE PATHS IN *N. crassa* AND *T. utilis*

Both *N. crassa* and *T. utilis* appear to have the following path of ornithine synthesis: glutamate, glutamic  $\gamma$ -semialdehyde, ornithine (see Fig. 2). It has, however, not been determined whether this route is the major one to ornithine in these organisms. The evidence for this path rests on the following findings obtained with mutant and tracer techniques. In *N. crassa*, glutamate has been found to give rise to glutamic  $\gamma$ -semialdehyde (10, 11) and to ornithine (11). Moreover, an enzyme has been extracted from this organism which can convert glutamic  $\gamma$ -semialdehyde to ornithine (15). In *T. utilis*, glutamate has also been shown to be a precursor of glutamic  $\gamma$ -semialdehyde (11) and of ornithine (8, 11). Furthermore, evidence has been obtained indicating that the two fungi do not produce ornithine via acetylated intermediates:  $N^{\alpha}$ -acetylornithine does not

<sup>4</sup> In analogy with ornithine transaminase (15) which appears to catalyze a reversible reaction.



satisfy the growth requirements of *N. crassa* mutants responding to ornithine (6), and does not contribute to arginine synthesis in isotopic competition experiments with wild-type *N. crassa* and *T. utilis* (11); and no acetylornithinase activity could be detected in extracts of wild-type *N. crassa* (10).

#### LINKS, NOT DIRECTLY INVOLVING GLUTAMATE, BETWEEN PROLINE AND ORNITHINE IN *E. coli*

A metabolic connection, other than the one involving glutamate as common precursor, between proline and ornithine has been reported for *E. coli* (12). Cultivation of *E. coli* in the presence of radioactive N-acetylglutamate was found to lead to labeling not only of ornithine, as referred to above, but also of proline and glutamate, the resulting specific activity of the latter two amino acids being small compared to that of ornithine (12, 13). The further finding that the proline was labeled considerably more strongly than the glutamate led to the conclusion that in *E. coli* a link other than glutamate exists between the main routes of proline and ornithine formation, and that this link constitutes a minor pathway of proline synthesis (12, 13). It seems possible that this minor path may involve the formation of glutamic  $\gamma$ -semialdehyde either by deacylation of N-acetylglutamic  $\gamma$ -semialdehyde or by  $\delta$ -transamination of ornithine (13).

A minor link between proline and *exogenous* ornithine has also been observed (13). In tracer incorporation experiments, exogenous ornithine was found to contribute not only to arginine, in harmony with other findings (5), but also to some extent to proline (13). In isotopic competition experiments with ornithine as tracer and unlabeled glutamic  $\gamma$ -semialdehyde as competitor, the semialdehyde largely suppressed the labeling of proline, but not of arginine (13). The quantitatively minor conversion of exogenous ornithine to proline was therefore concluded to proceed by the following path:

exogenous ornithine  $\rightarrow$  glutamic  $\gamma$ -semialdehyde  $\rightarrow$

$\Delta^1$ -pyrroline-5-carboxylic acid  $\rightarrow$  proline



The step from exogenous ornithine to glutamic  $\gamma$ -semialdehyde may involve a  $\delta$ -transamination, but other possibilities have not been ruled out. A simple reversal of ornithine synthesis would not appear to be sufficient to account for the conversion of exogenous ornithine to proline, since an *E. coli* mutant blocked between ornithine and N<sup>a</sup>-acetylornithine also carried out this conversion (13).

#### LINKS BETWEEN PROLINE AND ORNITHINE IN *N. crassa* AND *T. utilis*

As mentioned above, metabolic links not directly involving glutamate have also been found in *N. crassa* and in *T. utilis*. In particular, the possible interconversion of proline and ornithine has attracted interest (14, 15). Recently, it was found that ornithine readily forms proline in *N. crassa* (10, 11) and *T. utilis* (11), but proline does not readily form ornithine in either organism (10, 11). Moreover, in other relevant respects, the two organisms behaved so similarly (11) that there is little doubt that the same type of proline-ornithine interrelation occurs in both.

The finding that ornithine readily yields proline was based on mycelial pad experiments with *N. crassa* (10) and on tracer experiments with *N. crassa* and *T. utilis* (11). In both of these studies, the ornithine was supplied exogenously to the organisms. The question thus arose whether endogenous ornithine also contributes to proline synthesis.

The evidence obtained from studies with *N. crassa* mutants showed that the ready conversion of ornithine to proline occurs only when the ornithine is supplied exogenously (see Fig. 2), and that endogenous ornithine makes at most a minor contribution to proline synthesis (10). Thus, an examination of *N. crassa* mutants, which respond alternatively to glutamic  $\gamma$ -semialdehyde, proline, or ornithine, indicated (10) that they are blocked in the formation of glutamic  $\gamma$ -semialdehyde as proline precursor, but are able to produce ornithine from endogenous sources for conversion to its known metabolic products (25), citrulline and arginine. Consequently, this endogenous ornithine apparently is not available for adequate proline synthesis. Similarly, if the conversion of glutamic  $\gamma$ -semialdehyde to



ornithine is a quantitatively substantial reaction, glutamic  $\gamma$ -semialdehyde as ornithine precursor does not seem to be available for adequate proline formation. It may therefore well be that *N. crassa* is organized in such a manner that the paths of proline and ornithine synthesis are physically separated. It was suggested (10) that such a separation may be caused by a spatial organization of relevant enzyme systems,<sup>5</sup> with a resulting more or less restrictive "channeling" of metabolites.<sup>6</sup>

Independent evidence for channeling has been obtained from tracer incorporation experiments with *N. crassa* and *T. utilis* (11). When labeling was introduced into ornithine and proline through the Krebs cycle, the resulting specific activities of arginine (formed via ornithine) and of proline were approximately equal. In contrast, when the labeling was introduced by the use of exogenous glutamate, significant differences in specific activity were found in the arginine and proline formed.<sup>7</sup> These observations were interpreted as supporting the notion of a physical separation of the proline and ornithine paths (11).

Evidence was also presented that ornithine participates in a channeled path in *T. utilis* (11). In incorporation experiments, when labeled ornithine was supplied at a concentration of about 1 microgram per ml., the resulting specific activities of the proline and arginine formed were in the ratio 100:6 respectively; when the concentration of the labeled ornithine was increased to 50 micrograms per ml., this ratio was 100:80 respectively (11). These results indicate that the exogenous ornithine as source of proline does

<sup>5</sup> For a review on organized enzyme systems, see Green (26). This review deals primarily with particulate systems. The term "organization," as used here, is not intended to be necessarily restricted to so-called particle-bound enzymes.

<sup>6</sup> Thus, the enzyme system involved in the formation of glutamic  $\gamma$ -semialdehyde as proline precursor may differ, with respect to organization and possibly to other properties, from that thought to be involved in the formation of the semialdehyde as ornithine precursor. Also, the enzyme catalyzing the ready conversion of exogenous ornithine to glutamic  $\gamma$ -semialdehyde may differ, as to organization and perhaps as to other properties, from the enzyme thought to be involved in the biosynthetic conversion of glutamic  $\gamma$ -semialdehyde to ornithine.

<sup>7</sup> The extent of the labeling obtained may well be affected by the nature and size of possible glutamate reservoirs (27).



not equilibrate with the endogenous ornithine as source of arginine; however, the separation of the exogenous from the endogenous ornithine is seen to be by no means complete.

Moreover, the fact that exogenous differs from endogenous ornithine in their respective tendencies to form proline and arginine supports the view of a physical separation of the proline and ornithine pathways as distinct from the notion that a non-channeled common precursor may be utilized more effectively in one of the paths than in the other.

In view of the conclusion that in *N. crassa* (as well as in *T. utilis*) ornithine participates in a channeled pathway, it seems of interest that in *N. crassa* several genetically different ornithine-requiring mutants are known (25), the nature of whose blocks so far has not been determined. The possibility might merit consideration that some of these mutants are blocked through a disturbance in enzyme organization, rather than through some other change associated with an enzyme, or through the actual absence (cf. 15) of an enzyme. Such a disturbance might affect either the synthesis of ornithine, or even its utilization in the formation of arginine via citrulline. The latter possibility is conceivable, since for example a possible break in enzyme organization between ornithine and citrulline might interfere with the effective removal of ornithine from its site of formation. Such interference might result in a sufficiently large decrease in the rate of ornithine synthesis to cause a block. On the other hand, the mutants carrying such a block would be expected to be able to utilize exogenous ornithine. If this type of genetic block exists, the one-gene-one-function hypothesis (28) would still seem sufficiently elastic to embrace control of enzyme organization as one possible gene function.<sup>8</sup>

<sup>8</sup> For a review on gene function in relation to enzymes, see Bonner (29).

## COMPARATIVE BIOCHEMISTRY OF THE GLUTAMATE-PROLINE-ORNITHINE INTERRELATION

From the point of view of comparative biochemistry, it seems especially interesting that the glutamate-proline-ornithine interrelation found in *N. crassa* and *T. utilis* is closely similar to the one which was so elegantly shown in mammals by Stetten (30). On the other hand, this interrelation is seen to be quite different in *E. coli*. Thus, although all these organisms utilize glutamate, proline, and ornithine as metabolites, the pathways associated with these metabolites show differences. Such instances of biochemical diversity should prove useful in the study of taxonomic and evolutionary relationships. In this connection, it seems particularly striking that the two fungi more nearly resemble mammals than they do the bacterium *E. coli*. This observation by no means stands alone: for example, Dr. Yanofsky, elsewhere in this volume, presents evidence that the tryptophan-niacin relationship in *N. crassa* is similar to that in mammals but differs from that in bacteria.

Finally, a comment on the modes of ornithine formation in all of these organisms might be of some interest. If it is assumed that the ornithine path described here for the fungi is the major one leading to this amino acid, a generalization becomes possible.

It has been demonstrated that the transamination of glutamic  $\gamma$ -semialdehyde to form ornithine is a reversible reaction, with the equilibrium far on the side of the semialdehyde (15, 31). Presumably, the position of this equilibrium is affected by the tendency of the semialdehyde to cyclize. It appears that in the fungi, and possibly in mammals, a mechanism has been evolved, namely, channeling, which permits an adequate rate of ornithine synthesis, in spite of the unfavorable equilibrium involved. The channeling might achieve this effect either by counteracting the tendency of the semialdehyde to cyclize, or by facilitating the removal of ornithine from the site where the latter compound is formed, or by both.

*E. coli*, however, does not appear to have the enzymatic equipment for adequate production of ornithine from glutamic  $\gamma$ -semialdehyde.



This organism has instead evolved a mechanism, namely, the use of acetylated intermediates, which bypasses the equilibrium disadvantage imposed by the cyclization of glutamic  $\gamma$ -semialdehyde.

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## DISCUSSION

DR. COHEN: This session has concerned itself essentially with 3 areas which are not readily dissociable, and while identifiable as separate areas, are all intimately related. We have had 2 elegant presentations dealing with the urea cycle, a series of papers dealing with the arginine and citrulline breakdown in microorganisms and to a lesser extent in animal tissues, and, finally, the ancillary areas dealing with the components of these systems which are coupled, directly or indirectly, in the general metabolic scheme. I should like to introduce one historical aspect which I think is of some interest in retrospect. It is perhaps not appreciated that while there has been a recent resurgence of interest in arginine and citrulline breakdown, historically this reaction made it possible for Krebs to discover the urea cycle. It turns out that Ackerman in the early 30's was studying putrefaction and he had isolated a compound which, because of the work of Wada, he was able to identify as citrulline. The only supply of citrulline then available to Krebs was this small sample which Ackerman had isolated because of what we now recognize as the arginine dihydrolase or the citrulline phosphorylase system, present in the microorganisms of the putrefying system. It was from Ackerman that Krebs obtained the initial 20 milligrams of citrulline which permitted him to complete the study of the intermediate steps, ornithine to arginine. I think that from a historical standpoint this is of some interest. I would like to open this meeting for discussion, and to entertain questions directed at the speakers on this over-all topic. I will be very glad, if any of the speakers feel that they had their time so imposed upon that an additional comment or two is necessary, to entertain additional comments from them if they feel that it would add to this discussion.

DR. RATNER: I would like to mention in connection with the arginine desimidase reaction which Dr. Oginsky discussed that we have purified that enzyme quite extensively. We were interested in seeing if there were any cofactor requirements after extensive purification. We have not yet been able to find any requirements, but it would take too long to go into details on this.

DR. OGINSKY: We have done several ammonium sulfur fractionations and got high activity but obtained nothing at all with respect to cofactor requirements. We made 4 or 5 fractions by very gradual ammonium sulfur precipitation.

DR. RATNER: The other point is a question that I would like to ask Dr. Grisolia. I wonder what he thinks about the possibility of transferring a carbamyl group, enzymatically from Compound X to some acceptor, other than ornithine, for instance. I wonder whether you have looked for this.



DR. GRISOLIA: As a matter of fact, we have some information on this point. Let me say first, that we have been impressed by the complexity of the Urea Cycle; however, this might be due to other metabolic roles of the cycle in addition to urea production. We thought at the very beginning of our work, as you know, that there was a direct transcarbamylation reaction between carbamyl glutamate and ornithine to form citrulline. We looked quite hard for such a reaction as well as for more general biological transcarbamylation reactions involving amino acids. Eventually we came to the discovery of Compound X. We conducted some preliminary experiments to test for other acceptors for Compound X in addition to ornithine. The positive preliminary evidence on this point may give the reason, or one of the reasons, why the synthesis of citrulline is such a complicated reaction since in addition to forming citrulline, and thus urea, it appears that Compound X may play a central role in other reactions.

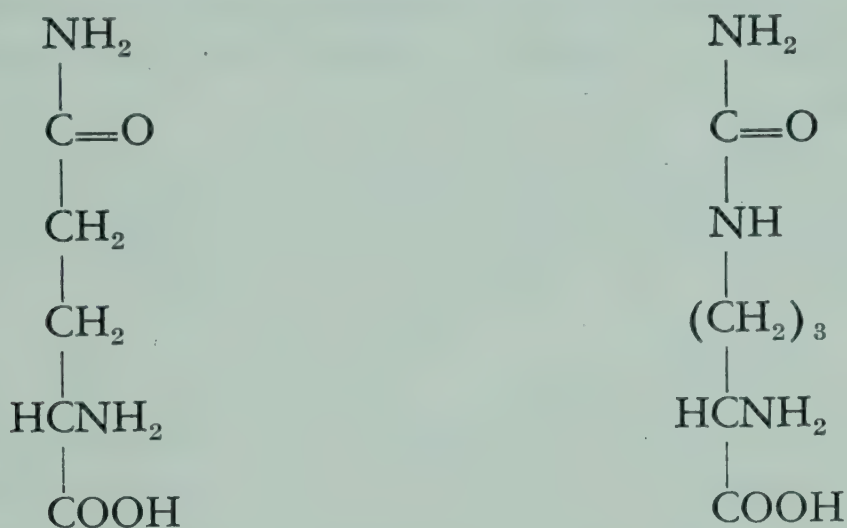
We have evidence indicating that there are at least two other acceptors in addition to ornithine. It appears at present that aspartate can be an acceptor for Compound X to form carbamyl aspartate. This is supported not only by some preliminary evidence which I have but also by evidence obtained by Dr. Peter Reichard of the Karolinska Institute. As a matter of fact I have here with me a letter of June 9, in which he informs me that he has what appears to be the separation of an enzyme system which activates Compound X and/or aspartate to form carbamyl aspartate. This enzyme system appears to be different from the enzyme system which uses ornithine and Compound X to form citrulline. I have sent the conditions for isolation of pure Compound X to Dr. Reichard, so as to test unequivocally his new enzyme, since up to now Dr. Reichard's experiments have been carried out by coupling his enzyme to a crude enzymatic system making Compound X. I have had permission to quote Dr. Reichard and I am most anxious to hear more of his beautiful work. From this discussion it is clear then, that we have another transcarbamylation reaction from Compound X and another interrelation with the biosynthesis of pyrimidines. Years ago, we conducted preliminary experiments which indicated other possible metabolic relations of the citrulline system with purine metabolism. I do feel there are other reactions involving Compound X but I am not prepared to talk about them now.

DR. COHEN: I might add one comment relating to the arsenolysis of citrulline. A similar effect has been demonstrated with carbamyl aspartic acid in liver tissue by Dr. Lowenstein following a discussion with Dr. Krebs. If one adds carbamyl aspartate to suitable liver preparations in the presence of arsenate one gets about  $\frac{2}{3}$  or  $\frac{1}{2}$  of the amount of  $\text{CO}_2$  that you would get under comparable conditions with citrulline. This has not been tried with microbial systems, but it does suggest the fact that the degradation of



carbamyl groups by the arsenolytic mechanism does in fact take place with other carbamyl compounds. There is considerably less degradation of carbamyl glutamate with these preparations in the presence of arsenate.

DR. LEVINTOW: I think it is of interest to point out certain similarities between the citrulline phosphorylase reaction, and some reactions involving glutamine, which is really a rather close structural analog of citrulline:



In the citrulline phosphorylase reaction, liberation of ammonia from the carbamido radical is associated with the synthesis of ATP from ADP and P. This situation is closely paralleled by the reversal of the enzymatic synthesis of glutamine, in which liberation of the amide ammonia is similarly associated with formation of ATP from ADP and P. (Levintow, L. and Meister, A., *J. Biol. Chem.* 209, 265 (1954)).

The purified enzyme which catalyzes this reaction also catalyzes a rapid arsenolysis of glutamine, another point of similarity with the citrulline phosphorylase system. The arsenolysis of glutamine, however, requires a divalent cation and catalytic amounts of ADP.

DR. KAPLAN: I was wondering, Dr. Grisolia, since you spoke of the acceleration of ATP breakdown by hydrazine and hydroxylamine, whether the glutamine-synthesizing enzyme is involved in your system. I ask this because hydrazine and hydroxylamine both can replace ammonia in the glutamine synthesizing system.

DR. GRISOLIA: I do not know. These preparations which are only partially purified do contain some activity for glutamine synthesis, but not very much. However, this is not terribly important because even in crude preparations we have never obtained any evidence for the activation of the carboxyl group of carbamyl glutamate as shown by the negativity of the hydroxamic test.

DR. NOVELLI: In that connection, I notice that acetyl glutamate was much more effective than carbamyl glutamate. I am wondering if you have tried alpha acetyl glutamate.



DR. GRISOLIA: No, we have never tried that. We have tested, however, a large number of compounds, among them carbamyl glutamine and carbamyl iso-glutamine neither of which can replace carbamyl glutamate. We have tried also formyl glutamine and iso-glutamine which are also inactive. So by analogy I do not think that the acetyl glutamine or iso-glutamine will be found to be active. However, I would like to test them.

DR. LIPMANN: I think you said something about the acetylation of glutamic acid. Did you have additional evidence on this? Could you tell us a little more about that?

DR. GRISOLIA: Yes, I took away the slide on that for two reasons: First, the evidence is very preliminary, and second, to shorten this presentation, so as to have more time for discussion as suggested by our Moderator. The evidence for this, which is very preliminary, rests on the coupling of acetyl glutamate synthesis with the citrulline-synthesizing system. Under our conditions we obtain about two micromoles of citrulline synthesis above the control values without glutamate or acetate, when we use glutamate and acetate together. This method, although conveniently simple, has two main weak points. First, it does not permit the unequivocal evaluation of acetyl glutamate synthesis, since this compound will react catalytically in the citrulline synthesizing system. Second, the optimum conditions required for acetyl glutamate synthesis are not desirable for citrulline estimation since, for example, small amounts of  $-SH$  compounds are extremely inhibitory for the colorimetric method used in citrulline estimation. Coenzyme A will also run you into trouble. Furthermore, at the present, this system that we have been using is so crude that the number of interfering reactions is very large; for example glutamine and acetyl glycine synthesis.

DR. WORK: I would like to ask Dr. Vogel what the evidence is for the acetylation of glutamic semi-aldehyde in *E. coli* and not in *Neurospora*.

DR. VOGEL: In *E. coli*, as I tried to point out, we know that glutamic acid is acetylated on the nitrogen first, and we also know from tracer experiments that the acetylglutamic acid goes to ornithine not via glutamate, but apparently in a direct manner, and we showed the intermediate steps. We isolated the acetylglutamic gamma-semialdehyde from the culture filtrates of an ornithineless mutant, so that establishes the participation of the acetyl semialdehyde in *E. coli*. In *Neurospora* we have several types of evidence against the participation of the acetylated intermediates, and one type is (for example) that  $N^a$ -acetylornithine is not a growth factor for any of a number of ornithine-requiring mutants that were tested. Another type of evidence comes from tracer experiments, and we could show that acetylornithine does not contribute any tracer to arginine in either *Neurospora* or *Torulopsis*; so we conclude that the acetylated pathway for ornithine does not exist here, although one cannot say from the evidence that the pathway has been ruled out critically.



DR. DAVIS: If I have understood him correctly, Dr. Vogel's conclusion, that the path of ornithine biosynthesis in *Neurospora* is different from that in *E. coli*, has been based on the observation that a compound that is an ornithine precursor (and an active growth factor for certain mutants) in *E. coli* has no growth-factor activity for ornithine auxotrophs of *Neurospora*. I would have reservations about accepting such an inference simply from lack of growth-factor activity, since this lack can be due to difficulty in penetration. As known examples of intermediates that lack growth-factor activity one might cite several of the intermediates in aromatic biosynthesis; the histidine precursors described by Dr. Ames; and citrate, which cannot replace glutamic acid in an *E. coli* mutant blocked in the citrate condensing reaction, but can do so in a similar mutant of so closely related a species as *Aerobacter aerogenes*.

DR. VOGEL: The conclusion that ornithine biosynthesis in *Neurospora* differs from that in *E. coli* rests on a number of observations, including some to which questions of penetration do not seem to apply. For example, our studies with extracts derived from the wild-type organisms show that *E. coli*, but not *Neurospora*, has appreciable acetylornithinase activity; and the tracer results reported indicate that *Neurospora*, but not *E. coli*, has appreciable ornithine  $\delta$ -transaminase activity. Thus, each enzyme appears to be associated with only one of these two organisms, and each enzyme is seen to be characteristic of its respective ornithine path. The conclusion drawn is therefore supported by the enzyme studies as well as by the mutant and tracer studies discussed before.

DR. SLADE: With regard to citrulline arsenolysis, from the various species of organisms that have been used by Dr. Oginsky, Dr. Knivett and myself, we found that the rate of the arsenolysis reaction is somewhat close to the rate found with the system in the presence of phosphate. It may be two times the rate of the phosphate reaction in some cases, but Dr. Korzenovsky in some of his slides showed a very high activity of the order of 125 in arsenate systems as compared with 8 in the phosphate systems. I was wondering whether you still have the effect of fluoride inhibition.

DR. KORZENOVSKY: Yes.

DR. SLADE: I was wondering if anyone wants to comment here on the possibility that this is a spontaneous decomposition of an arsenylated intermediate and, if so, how it can be so fast in one species and of an entirely different order of magnitude in other species.

DR. KORZENOVSKY: I might just say that in order to show this increased activity in the presence of arsenate we have had to use an enzyme preparation that was free of phosphate, since phosphate is inhibitory to the arsenolysis reaction. The other thing is that, in addition, we have found that the



affinity for arsenate is quite low. Therefore, it was necessary to use quite high concentrations of arsenate to obtain these effects.

DR. GRISOLIA: Since we have found recently this new enzyme, or enzymes, of which I spoke today which decomposes Compound X, apparently by initial cleavage of the phosphate group, it may be that differences in some of these reactions would be explained by the participation in arsenolysis of Compound X or related compounds.

DR. HANDLER: I would like to point out that Dr. Vogel apparently believes his dictum.<sup>1</sup> I do not believe that Dr. Stetten directly established the existence of the aldehyde intermediate in mammalian metabolism. Is this correct?

DR. VOGEL: Dr. Stetten demonstrated the conversion of proline to ornithine.

DR. HANDLER: Yes, unquestionably; but did she establish the aldehyde as an intermediate?

DR. VOGEL: Well, this is the situation. Dr. Stetten had a paper in the *Journal of Biological Chemistry* in 1951 where she studied the conversion of ornithine, labeled specifically in the alpha or delta nitrogen, to glutamate and to proline, and she was able to establish that the main attack on the ornithine, the primary or initial attack, was on the delta amino nitrogen, and this appeared to contribute to the glutamate. The residual semialdehyde then goes to proline, and she showed that the proline had more of the alpha nitrogen.

DR. HANDLER: But she never handled the semi-aldehyde or the pyrroline compound!

DR. VOGEL: No. She never handled the aldehyde. As a matter of fact, the first mention of the actual handling of the aldehyde, I believe, was by Taggart and Krakaur, who showed it to be a degradative product of proline oxidation, and isolated it as a 2,4-dinitrophenylhydrazine derivative. This gave the correct analysis, but unfortunately there is reason to believe that the compound was not, at least not exactly, what they thought, because it was an infusible compound. Glutamic gamma-semialdehyde was synthesized by myself and Dr. Davis, and its diethylacetal by Good and Mitchell. Good and Mitchell prepared the 2,4-dinitrophenylhydrazone of DL-glutamic gamma-semialdehyde, and that showed a melting point. I don't think it's likely that the natural compound should be infusible, so I think it is possible that Taggart and Krakaur had some other compound. By this I do not mean to indicate that their conclusion is wrong. They have other evidence, and I am certain that they had glutamic semialdehyde, but it actually didn't become available as such until it was shown to be accumulated by *E. coli* mutants and

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<sup>1</sup> EDITORS' NOTE: The dictum: "If one wants to study mammalian biochemistry it might be better to work with *Neurospora* than with *E. coli*."



synthesized from gamma, gamma-dicarbethoxy-gamma-acetamidobutyraldehyde. Coming back to your original question, just to answer it very briefly, Stetten did not have the semi-aldehyde, but her results fitted in perfectly with its existence which she postulated, and Dr. Meister's finding that there is a mammalian ornithine delta-transaminase seems to support this very well.

DR. HANDLER: Some years ago Bernheim reported that caffeine would inhibit the synthesis of urea in liver slices, and he also did some work with the intact rat and observed the same inhibition. If I recall correctly, this inhibition could be overcome equally well by glutamine or ornithine. I wonder if you know where in this series of reactions caffeine might be operating.

DR. GRISOLIA: It is my recollection that in earlier work Drs. Cohen and Hayano tested the effect of caffeine and found inhibition at the ornithine → citrulline step. Whether or not it acts on the synthesis of Compound X we do not know.

DR. HANDLER: Which step is affected?

DR. COHEN: It's in the first step. Dr. Hayano didn't study this in great detail, but as I recall there was a considerable order of inhibition in these crude preparations. It hasn't been studied with the purified enzyme.

DR. HANDLER: Has it been determined why glutamine overcame the caffeine inhibition?

DR. COHEN: The effect of glutamine in overcoming that inhibition was not checked.

DR. MEISTER: I wonder if Dr. Vogel or anyone else interested in this area would care to comment on the possible role of delta-1, pyrroline-2, carboxylic acid. I have heard all of this session, and no one has mentioned this compound, although it has been mentioned in the literature.

DR. VOGEL: I would like to answer that, Dr. Meister, if I may. I am aware of your work, and I am also aware of the fact that you elegantly synthesized the alpha-keto analogue of ornithine and showed that it cyclizes to form the pyrroline compound which you mentioned, which reacts with ortho-amino-benzaldehyde, and so on. This I was going to give in the introduction, but in order to save time I left out the first slide; but I am happy to correct this omission now. Dr. Stetten and others have concluded that although enzymes which can catalyze the formation of the alpha-keto analogue of ornithine are widespread in nature, there is no evidence or any reason to believe that this keto analogue of ornithine plays any role in the actual interrelation among glutamate, ornithine, and proline. If I remember correctly, Dr. Meister, I believe you showed that delta<sup>1</sup>-pyrroline-2-carboxylate is in fact inactive for a number of *E. coli* mutants which you tested, and this compound is readily distinguishable from delta<sup>1</sup>-pyrroline-5-carboxylate.





## Part III

*METABOLISM OF HISTIDINE, LEUCINE, ISOLEUCINE,  
VALINE AND LYSINE*





# THE BIOSYNTHESIS OF HISTIDINE

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## NUTRITIONAL AND TRACER STUDIES

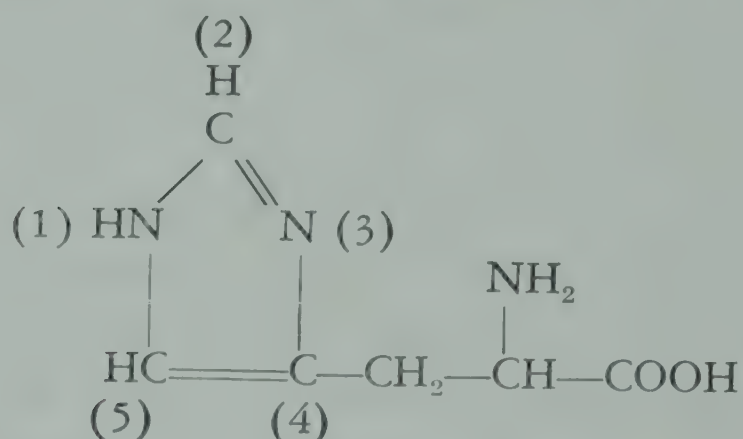
INTEREST IN HISTIDINE synthesis dates back to early nutritional work showing histidine to be an essential amino acid for growing animals. These and later animal growth experiments furnished information on the ability of various animals to convert related compounds to histidine. The rat, for example, will use N<sup>α</sup>-acetyl histidine (20), D-histidine, imidazole lactic acid, or imidazole pyruvic acid instead of L-histidine (7, 8, 13). There is no evidence that any of these compounds can be formed from compounds other than histidine in vivo. These studies, therefore, provide no evidence concerning a pathway of net synthesis. For an understanding of the de novo pathway of biosynthesis, plants or microorganisms which normally do not require histidine have been studied. Aside from tracer work, most of the investigations on these histidine-independent organisms have involved blocking the normal synthetic pathway by some means.

*Pyridoxal—histidine interactions.* Broquist and Snell (5) found that *Lactobacillus arabinosus* required histidine for growth only when pyridoxal was omitted from the culture medium. This suggested that one of the steps in the biosynthesis was catalyzed by pyridoxal phosphate. The conversion of imidazole pyruvic acid to histidine is dependent on a pyridoxal phosphate enzyme in *Streptococcus faecalis* and in other organisms. These observations taken together led them to postulate that imidazole pyruvic acid was an intermediate in the biosynthesis of histidine.

\* Postdoctoral fellow of the United States Public Health Service.



The role of purines and formate in the synthesis of the imidazole ring. Broquist and Snell found an increased requirement for purine when *Lactobacillus casei* was grown without histidine. They suggested that purines are precursors of histidine. A partial clarification of the role of purines in histidine synthesis as well as the origin of the amidine carbon of the ring (carbon 2) resulted from the



experiments of Levy and Coon (15) using  $\text{C}^{14}$ -labeled substrates. These investigators grew yeast (*Saccharomyces cerevisiae*) on a medium containing glucose and a small amount of formate- $\text{C}^{14}$  as essentially the only carbon sources. The amino acids from the proteins were fractionated on an ion exchange column. Only histidine had appreciable radioactivity and this was all in the 2-carbon atom. Similar experiments were done with glycine-1- $\text{C}^{14}$  in place of the formate in one case, and bicarbonate- $\text{C}^{14}$  in the other. In the glycine and bicarbonate experiments, histidine had negligible radioactivity. These experiments indicate that the 2-carbon atom of histidine comes fairly directly from formate, and that glycine is not involved in the synthesis of the imidazole ring in histidine. Yeast has been shown to incorporate glycine directly into the purine imidazole ring. The non-incorporation of glycine into the histidine imidazole ring indicates that this ring is synthesized by a different mechanism and that the purine imidazole ring is not a precursor of the histidine imidazole. Tabor et al. (23), working with the yeast *Torulopsis utilis*, also found that formate- $\text{C}^{14}$  is incorporated into the 2-carbon of histidine. Purine catabolism is known to furnish formate, and a possible explanation of the results of Broquist and Snell is that purines serve as formate donors. It would be of interest

to see whether formate is equivalent to purines in sparing the histidine requirement of *L. arabinosus*, and whether pyridoxal is still required if this is the case.

*The origin of the main carbon chain.* Levy and Coon (16) have presented a preliminary report on their experiments designed to find the origin of the five-carbon chain of histidine. They grew *Saccharomyces* on uniformly labeled glucose and non-labeled glutamic acid in an attempt to find out if glutamic acid is a fairly direct precursor of the five-carbon chain. The isolated histidine was highly radioactive in the five-carbon chain, a result indicating that histidine does not come from glutamic acid or its metabolic products, and might come fairly directly from glucose. They also did a similar experiment with methyl-labeled acetate. Acetate apparently is not directly involved in the synthesis of the principal carbon chain of histidine.

#### THE HISTIDINE MUTANTS OF NEUROSPORA

Another approach to the problem has been through biochemical genetics, by using genetic blocks to analyze a metabolic pathway. I would like to present the studies on a series of histidine-requiring mutants of *Neurospora crassa* in a more or less chronological order, as this illustrates some of the advantages and drawbacks of the mutant methods. This work covers the establishment of a serial order of the mutants through a genetic analysis, the characterization of inactive substances accumulated by the mutants, the isolation of phosphate esters of these compounds (apparently the true intermediates), and the enzymatic interconversion of these phosphate esters.

A series of histidine-requiring mutants of *Neurospora* was isolated by various workers in the Mitchells' laboratory at the California Institute of Technology. These were analyzed genetically by Dr. Felix Haas and Mary Mitchell (12) and were found to fall into five genetic groups. One mutant from each group was used for the subsequent biochemical and genetic studies.

*Specificity of the histidine requirement.* The author, in conjunc-



tion with the Mitchells and Haas, carried out a study of the specificity of histidine as a growth-promoting substance early in the biochemical investigation of the *Neurospora* mutants. We tried a large group of natural and synthetic imidazoles on the mutants with no success. These included imidazole lactic acid, 4-amino-5-carboxamide imidazole, and all the simple imidazoles with side chains at the 4(5) position, as well as all the common purines and amino acids.

*Accumulations by the mutants.* A characteristic of some biochemical mutants which makes them very useful for the elucidation of biosynthetic pathways is the accumulation of precursors which pile up because of the blocked reaction. Accumulations have been demonstrated in certain tryptophan, purine, pyrimidine, choline, thiamine, aromatic, and other biochemical mutants (14, 17). These precursors can often be assayed by their activity for other mutants blocked at an earlier reaction step. The information gained from accumulations is not always clear, however, as some accumulated precursors are modified by normal metabolic reactions. For example, a *Neurospora* nicotinic acid mutant blocked in the conversion of kynurenine to hydroxykynurenine accumulates N<sup>a</sup>-acetylkynurenine and kynurenic acid, two metabolically inactive derivatives of kynurenine (14). Undoubtedly the equilibrium between various accumulated precursors is also affected by numerous secondary metabolic factors.

In collaboration with Haas, an attempt was made to demonstrate the accumulation of active intermediates by the histidine mutants (12). None of the mutants was observed to accumulate any substance in the medium which would stimulate the growth of any other histidine mutant.

In the early work with mutant C84, which was the first histidine mutant isolated, Mitchell and Mitchell found that a substance was accumulated in the culture medium that reacted to form a red dye with diazosulfanilic acid, a reagent which Pauly (22) used for detecting imidazoles. When we adapted this reagent for paper chromatography (2) and chromatographed the culture filtrates, it was found that mutants C84, C141, and T1710 accumulated imidazoles as shown in Fig. 1. Wild-type *Neurospora*, mutant C94, and

mutant C140 did not accumulate these compounds. Mutants C84 and C141 seemed to accumulate one compound in common. These three compounds were not chromatographically identical with any of the known imidazoles used as standards.

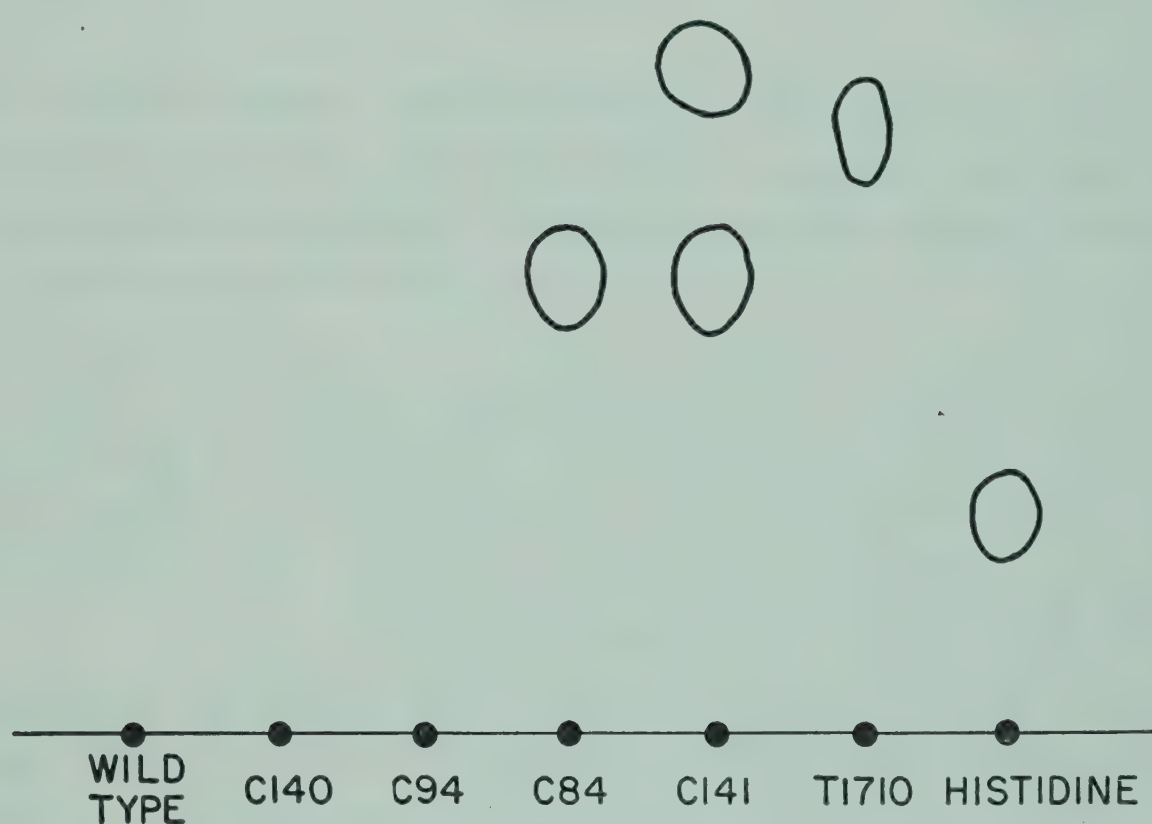
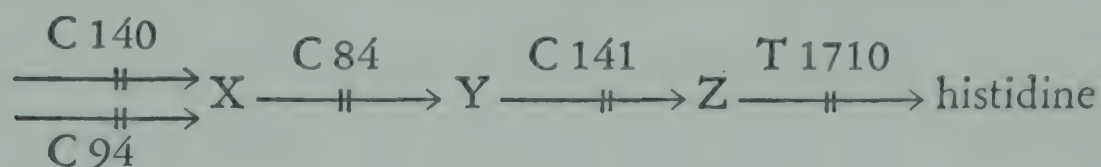


FIG. 1. Chromatogram of mutant extracts run in 3:1 propanol: 1 N ammonia. The chromatogram was developed with a diazo spray for imidazoles (2).

*Serial order of the mutants.* Previous investigations in Mitchell's laboratory had made use of double mutants to provide evidence for a biochemical sequence for *Neurospora* mutants that have the same growth requirement (18, 19). A compound accumulated by a mutant because of the genetic block will no longer be produced if a double mutant is made by introducing a gene that blocks a reaction step earlier in the biosynthetic sequence. Haas, in analyzing the genetics of the mutants, had made the double mutants, and we examined these for accumulations, both chromatographically and quantitatively. Based on the evidence from these double mutants, and on



the assumption that these compounds were histidine precursors or derivatives of histidine precursors, an order of the mutants in the biosynthetic scheme was postulated. C141 was placed before T1710 in the sequence because the double mutant C141-T1710 behaved like C141 in accumulating X and Y rather than like T1710 in accumulating Z. The sequence was pictured as follows:



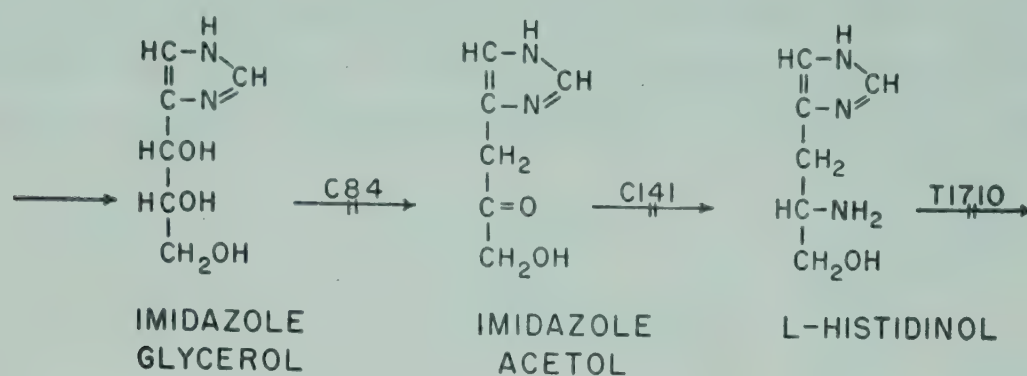
Genes C140 or C94 when introduced into a strain with any of the other genes stop all accumulations, so they were placed before the other three mutants in the sequence. By this analysis it was not possible to say whether C140 was ahead of C94 or vice versa.

*The characterization of the accumulated imidazoles.* Even though these compounds did not seem to be active growth factors for the mutants, their characterization was undertaken in the hope that this would tell us something about the biosynthesis. This work was done in collaboration with Mitchell and Mitchell.

At about this time Vogel, Davis, and Mingioli (25) isolated the very interesting compound L-histidinol from an *Escherichia coli* histidine mutant and observed that this compound was slowly used by another mutant in place of histidine. This was the first clue as to the nature of the synthetic pathway. They kindly sent us a sample of the compound, and this was shown to be identical with the imidazole accumulated by T1710.

The two compounds accumulated by C141 were isolated by means of their mercury salts and separated on a Dowex-50 column. They reacted with periodate, indicating a glycol or similar groupings. These compounds were crystallized and proven to be imidazole glycerol and imidazole acetol (4), compounds not previously described. Imidazole glycerol was also accumulated by mutant C84. [Formula A]. On treatment with periodate, imidazole glycerol gave the known compound imidazole formaldehyde and one equiva-

lent each of formic acid and formaldehyde. Imidazole acetol under the same conditions yielded imidazole acetic acid and formaldehyde.



FORMULA A

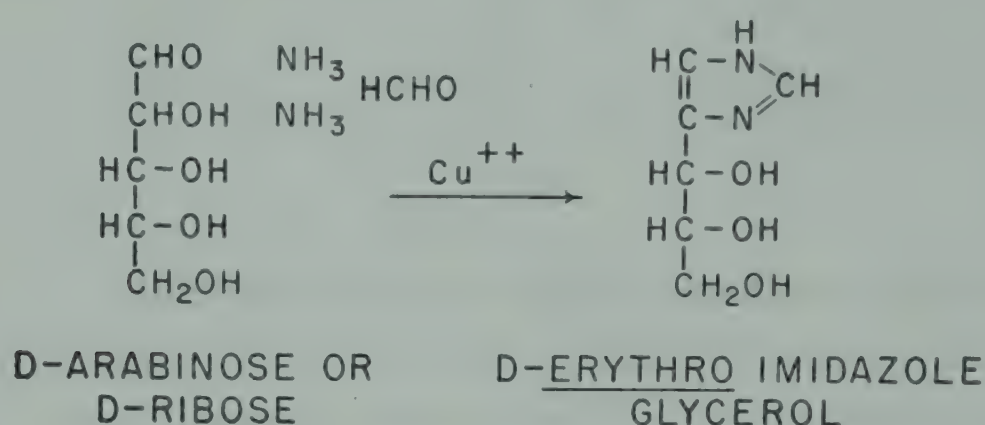
*The chemical synthesis of imidazoles from sugars.* I next digress a bit to discuss an interesting reaction which we had been toying with during the course of this work and which turned out to tie in with the *Neurospora* imidazoles.

The first synthesis of imidazole was by Debus (9), who prepared it from glyoxal, ammonia, and formaldehyde. He proposed the name glyoxaline for the compound, and this name is still used by the British chemists. In 1905, Windaus and Knoop (26) found that among the products formed when D-glucose or other ~~sugars~~ were allowed to react with a solution of zinc hydroxide in ammonia was the insoluble zinc salt of 4-methyl imidazole. They eventually formulated the reaction as involving the breakdown of the sugar in the alkaline solution to methyl glyoxal and formaldehyde and the condensation of these with ammonia to form the methyl imidazole.

In the 30's, Parrod (21) investigated the reaction further and found that by using cuprammonium solution he could isolate the copper salts of imidazole, hydroxymethyl imidazole, and 4-(D-arabo-tetrahydroxybutyl) imidazole from fructose, glucose, mannose, or D-glucosone. We prepared the tetrahydroxybutyl compound and also ran a similar sort of reaction with various pentoses. [Formula B]. All the reaction mixtures were tried out on the mutants in growth experiments with no success. Chromatography of both synthesis mixtures indicated at least four major substances, the main products being imidazole and hydroxymethyl imidazole. When one of the compounds from *Neurospora* turned out to be a trihydroxypropyl imi-



dazole, the pentose reaction mixtures were reexamined, and were found to contain a compound with the same R<sub>f</sub> value. The various isomers were synthesized from the different pentoses and the *Neurospora* compound was shown to be identical with D-erythro-trihydroxypropyl imidazole, which could be synthesized from D-arabinose or D-ribose (3).

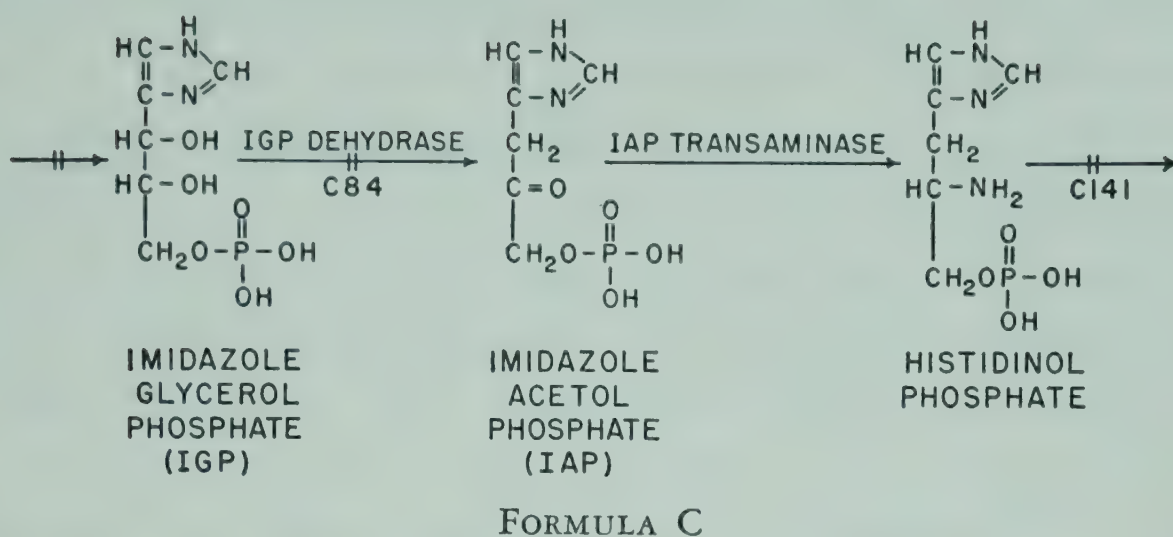


FORMULA B

*The accumulation of phosphate esters by the mutants.* The general picture of histidine biosynthesis seemed reasonable at this point, except that none of these compounds was active in replacing the histidine required by the mutants. Considering the structure of these compounds, permeability did not seem to be a good explanation for this lack of biological activity. One possible explanation was that the actual intermediates were more complex molecules to which *Neurospora* mycelium was not permeable. This led to a search for compounds of this type.

In the isolation procedures we have been using with these imidazoles, an extract of the mycelium had been combined with the growth medium before precipitating the accumulated compounds. Several compounds which gave an imidazole test and were present in smaller amounts were observed in the Dowex eluate. These were run on chromatograms before and after acid hydrolysis. Two of these compounds were shown to contain phosphate and to be hydrolyzed to imidazole glycerol and imidazole acetol, respectively (4).

Imidazole glycerol phosphate (IGP), imidazole acetol phosphate (IAP) and histidinol phosphate have now been isolated from the mycelium of C141, purified, and characterized (3). C84 accumulates imidazole glycerol phosphate [Formula C].



When a hot water extract of the mycelium of mutant C141 is chromatographed on a Dowex-1 formate column and the eluate is analyzed by the diazo test, the pattern in Fig. 2 is observed. Tyrosine

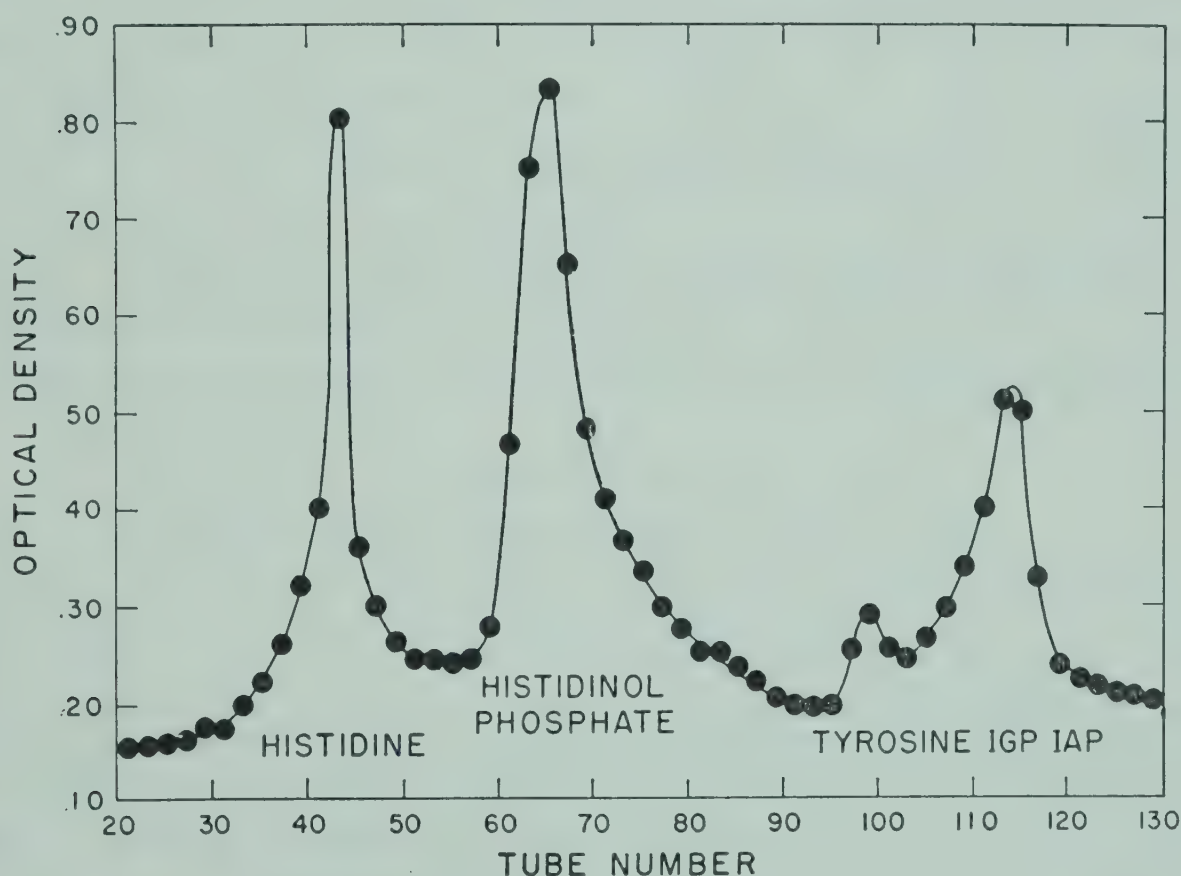


FIG. 2. Hot water extract of mutant C141 mycelium run on Dowex-1 formate column. Gradient elution using 2 liters water and 0.2 *N* ammonium formate (pH 5.0). The tubes were assayed with diazo reagent (3). The column was pretreated with pH 9 ammonia water to insure the binding of the histidinol phosphate.

gives a diazo color and appears as a small peak preceding the IAP + IGP peak. The imidazole acetol phosphate and imidazole glycerol phosphate peaks overlap in this first separation but can be resolved, by rechromatographing, on a Dowex-1 chloride column. All three



esters were still further purified by gradient elution from Dowex-1 chloride columns.

*Imidazole glycerol phosphate.* Extreme resistance to acid hydrolysis is shown by imidazole glycerol phosphate, as would be expected if the phosphate group were on the primary hydroxyl. Conclusive evidence that the phosphate is on the primary hydroxyl is furnished by the result of periodate oxidation. The phosphate ester, like imidazole glycerol, is cleaved by sodium metaperiodate to yield imidazole formaldehyde. Esterified glycols are not cleaved by periodate, so that the phosphate group cannot be on the secondary hydroxyls. This quantitative oxidation may be used as for determining these two compounds, as imidazole formaldehyde has a strong ultraviolet spectrum (24), unlike imidazoles that have no conjugation with the ring. On treatment with acid or phosphatase, imidazole glycerol phosphate is hydrolyzed to inorganic phosphate and imidazole glycerol. The nitrogen-phosphorus ratio is 1.9.

*Imidazole acetol phosphate.* Imidazole acetol appears upon hydrolysis of imidazole acetol phosphate by phosphatase or acid. The ester is easily hydrolyzed by acid, as are other  $\alpha$ -keto phosphate esters.

TABLE 1  
HYDROLYSIS OF IAP IN 0.1 N HCL AT 100° C.

Hours	1.5	2.5	5.5	9.5
Per cent hydrolyzed	32	41	71	95
Inorganic phosphate: imidazole acetol ratio	1.0	1.1	1.0	1.1

Inorganic phosphate was measured by the Fiske-Subbarow procedure (11) and imidazole acetol by the 370 m $\mu$  absorption in alkaline solution.

One equivalent of imidazole acetol is liberated for each equivalent of inorganic phosphate formed during the course of this hydrolysis (Table 1). It was found that imidazole acetol would enolize in strong alkali and that the conjugation with the imidazole ring resulted in an ultraviolet absorption spectrum similar to that of compounds such as urocanic acid (imidazole acrylic acid). The time curve of this enolization is shown in Fig. 3. A maximum is observed

at 370  $m\mu$  with an  $\epsilon$  of 10,400. The slow disappearance of the 370  $m\mu$  absorption may be due to polymerization or cyclization of the enol. The phosphate ester group seems to prevent enolization with the methylene carbon atom, and the small absorption of IAP is probably due to a slow hydrolysis in the alkali. The measurement of the imidazole acetol liberated after complete hydrolysis of the ester by acid (Fig. 3) serves as a sensitive assay for IAP.

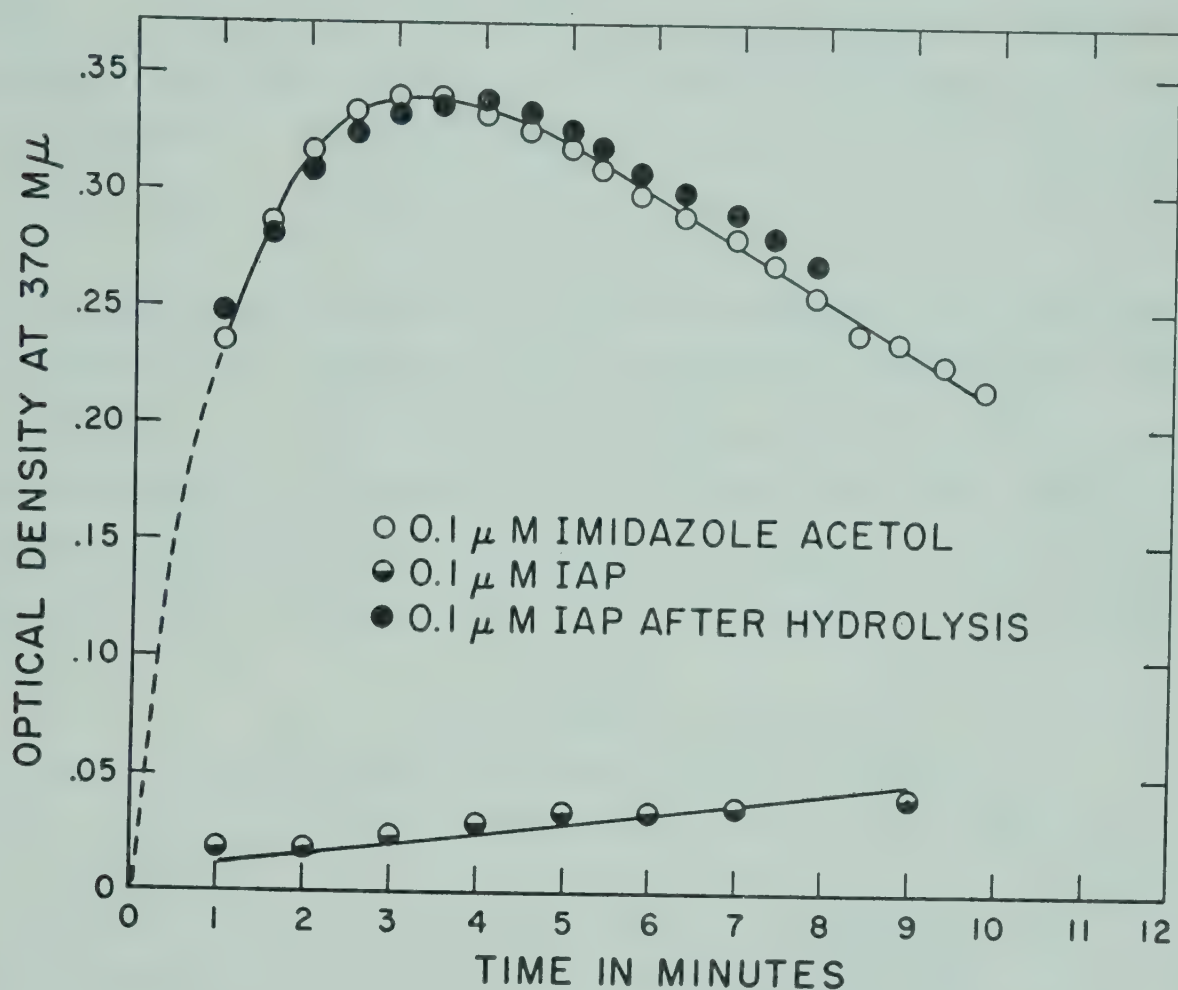


FIG. 3. 3 ml. of 3 *N* sodium hydroxide were added to 0.1 ml. of imidazole acetol at zero time. 0.2 ml. IAP and 0.1 ml. concentrated HCl were heated for 30 minutes on a steam bath to hydrolyze the ester. The alkali was added after cooling the tube.

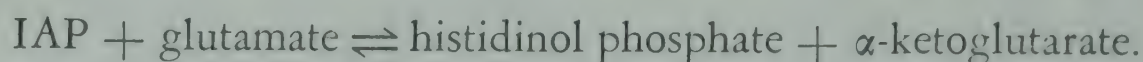
*Histidinol phosphate.* Mutant C141 was found also to accumulate another phosphate ester in the mycelium. This has been identified as L-histidinol phosphate. C141 was originally thought to be blocked before this step, but it now appears that it must be blocked after L-histidinol phosphate. Histidinol phosphate yields L-histidinol when hydrolyzed with acid or phosphatase and is identical with synthetic L-histidinol phosphate made by phosphorylating L-histidinol. The hydrolysis curves of histidinol phosphate in acid, alkali, and at pH



4.5 are similar to those reported for ethanolamine phosphate by Cherbuliez and Bouvier (6). Both these compounds are extremely stable to hydrolysis and are more easily hydrolyzed at a pH of about 4.5 than in 1 N acid or 1 N alkali.

None of these esters are active in replacing histidine for the mutants but, as has been pointed out, *Neurospora* mycelium is impermeable to phosphate esters.

*Enzymatic interconversion of the phosphate esters.* Recently, working in conjunction with Dr. B. L. Horecker, an enzyme has been obtained from *Neurospora* which catalyzes the reaction:



The reaction was first discovered by chromatographing incubation mixtures of histidinol phosphate and  $\alpha$ -ketoglutarate with a C141 acetone powder. After incubation the histidinol phosphate spot is almost gone, and new spots corresponding to imidazole acetol phosphate and to glutamate are evident. Wild-type *Neurospora* and mutant T1710 have also been shown to contain the enzyme.

TABLE 2  
IMIDAZOLE ACETOL PHOSPHATE TRANSAMINASE

	$\mu\text{M}$ IAP formed
Complete system	0.20
— histidinol phosphate	0.01
— $\alpha$ -ketoglutarate	0.00
— enzyme	0.00
— histidinol phosphate 0.7 $\mu\text{M}$ histidinol	0.00
— $\alpha$ -ketoglutarate + 6 $\mu\text{M}$ sodium pyruvate	0.03

The complete system contained 0.29  $\mu\text{M}$  of histidinol phosphate, 6  $\mu\text{M}$  of  $\alpha$ -ketoglutarate, 0.04 ml. treated enzyme, and 8  $\gamma$  pyridoxal phosphate. The mixture in pH 7 phosphate buffer was incubated two hours at 35° C. The enzyme was prepared by grinding 500 mg. of C141 acetone powder, 500 mg. charcoal, and 500 mg. Dowex-1 chloride in 10 ml. of pH 7 phosphate buffer and filtering.

The requirements of the system are shown in Table 2. No reaction was observed if  $\alpha$ -ketoglutarate, enzyme, or histidinol phosphate was left out of the mixture. The imidazole acetol phosphate was measured by the 370  $\text{m}\mu$  absorption after hydrolysis. The isolated

and synthetic histidinol phosphate are equivalent in the system, while histidinol does not react. Pyruvate will replace  $\alpha$ -ketoglutarate to a slight extent; however, a glutamate-pyruvate transaminase is known to be present in *Neurospora* (10). The disappearance of imidazole acetol phosphate in the presence of glutamate is shown in Table 3.

TABLE 3  
IMIDAZOLE ACETOL PHOSPHATE DISAPPEARANCE

	$\mu M$ IAP
complete system	0.13
— glutamate	0.20
— enzyme	0.19

The complete system contained 0.20  $\mu M$  IAP, 12  $\mu M$  glutamate and 2.5 mg. acetone powder. The incubation mixture in pH 7 phosphate buffer was kept for one hour at 35° C.

Treatment of the enzyme with Dowex-1 chloride and charcoal results in a partial pyridoxal phosphate requirement for the system (Table 4).<sup>1</sup>

TABLE 4  
PARTIAL RESOLUTION OF TRANSAMINASE

	$\mu M$ IAP	
	Exp. 1	Exp. 2
untreated enzyme, complete system	0.22	
— pyridoxal phosphate	0.22	
treated enzyme, complete system	0.20	0.20
— pyridoxal phosphate	0.13	0.11

The complete system and conditions are the same as in Table 2. In the untreated enzyme preparation, the charcoal and Dowex were omitted.

The reaction is an interesting one from the point of view of the mechanism of action of pyridoxal phosphate transaminases, as a phosphate ester replaces the usual carboxyl group. Perhaps this accounts for the phosphorylated compounds being intermediates rather than the free alcohols, though this explanation may be more of the chemist's prejudice than the mold's.

<sup>1</sup> We are indebted to Drs. Sober and Peterson of the National Cancer Institute for a sample of crystalline pyridoxal phosphate.



Chromatographic evidence has also been obtained for an enzyme from C141 which converts imidazole glycerol phosphate to imidazole acetol phosphate. This system is inactivated by ethylenediamine tetraacetate, and is presumably metal-dependent.

### DISCUSSION

The accumulation of these esters by the various histidine mutants and the evidence for their enzymic interconversion points strongly to their being precursors of histidine in *Neurospora*. The investigation of Vogel, Davis, and Mingioli on histidinol utilization by *E. coli* (25) and recent work of Elijah Adams (1) on a histidinol-oxidizing enzyme point to histidinol as a normal precursor of histidine in several organisms. Adams has purified a DPN enzyme from yeast, *Arthrobacter* and *E. coli* which specifically oxidizes L-histidinol to histidine. This enzyme is not present in those histidine-requiring coli mutants which will not utilize histidinol for growth.

Histidinol phosphate, therefore, seems a likely precursor of histidinol on the pathway of histidine synthesis. One disturbing observation is that none of the *Neurospora* mutants will use histidinol to replace their histidine requirement. This may be a question of permeability, or, what seems less likely at the moment, an indication that in *Neurospora* the phosphate group stays on during the final oxidation. Mutant T1710, which accumulates histidinol, contains a phosphatase which is very active in splitting histidinol phosphate, and mutant C141 splits this phosphate ester to some extent. As purified potato phosphatase also hydrolyzes the compound, the question of a specific hydrolysis enzyme is still open.

It would appear that mutant C141 is blocked somewhere between histidinol phosphate and histidine, and that mutant C84 is blocked between imidazole glycerol phosphate and imidazole acetol phosphate. The understanding of these blocks on a biochemical level will have to be deferred until further enzyme work is done.

The observation of Broquist and Snell that *L. arabinosus* required either pyridoxal or histidine for growth suggests that pyridoxal phosphate is a coenzyme for one of the steps in the biosynthesis.



Perhaps imidazole acetol phosphate transaminase is this enzyme, rather than imidazole pyruvic transaminase, which was originally postulated. The pyruvic transaminase seems to be widespread and occurs in many organisms which cannot synthesize histidine. Its function may be involved with histidine racemase activity rather than with histidine biosynthesis. If the pyridoxal-mediated reaction in *L. arabinosus* is the imidazole acetol phosphate transaminase, it will be of interest to see whether *L. arabinosus* can convert L-histidinol to histidine and whether any imidazoles are accumulated on a pyridoxal-deficient medium.

The nature of the compounds preceding imidazole glycerol phosphate on this scheme, and the mechanism of formate and nitrogen incorporation are still obscure. The D-erythro configuration of the IGP suggests that D-ribose-5-phosphate or D-ribulose-5-phosphate, which have this configuration and are of metabolic importance, might be the sugar precursors of histidine. In closing, I would like to quote M. Guggenheim's speculation on this point. In *Die Biogenen amine* (1940) he had this interesting comment on the Parrod reaction of sugars and  $\text{NH}_3$ : "Es erscheint nicht ausgeschlossen, dass auch in der lebenden Zelle eine ähnliche Reaktionsfolge von einer Pentose oder Hexose aus zur Bildung des Histidine führt."

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# DEGRADATION OF HISTIDINE

HERBERT TABOR

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ALTHOUGH HISTIDINE can undergo a number of metabolic transformations, most of the histidine administered to animals is com-

<b>C<sup>14</sup>O<sub>2</sub> from C<sup>14</sup>-Histidine Following Intravenous Injection in Mice</b>	
<b>Time</b>	<b>Expired CO<sub>2</sub> (cumulative) %</b>
10 Min.	1.3
20 "	9.3
30 "	26.1
1 Hr.	35.6
2 "	39.8
4 "	43.3
<b>(Borsook et al.)</b>	

FIG. 1. Cumulative excretion of C<sup>14</sup>O<sub>2</sub> after the intravenous administration of L-histidine-2-C<sup>14</sup>-imidazole (H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, 11). Similar data have been reported for C<sup>14</sup>O<sub>2</sub> excretion after histidine-C<sup>14</sup>OOH by D'Iorio and Bouthillier (18) and Novak (47) and after histidine- $\alpha$ -C<sup>14</sup> by Wolf (98).

pletely degraded to CO<sub>2</sub>. Thus, following the administration of C<sup>14</sup>-histidine, there is a rapid excretion of C<sup>14</sup>O<sub>2</sub> (Fig. 1). This review is mainly concerned with the intermediary steps in the degradation of histidine.



## MECHANISM OF HISTIDINE DEGRADATION

*Early studies on the enzymatic degradation of histidine.* The first demonstration of the enzymatic breakdown of histidine by liver preparations was presented in 1926 by Edlbacher (19) and by György and Röthler (25). This was followed by an extensive series of studies over the next twenty years by Edlbacher and his colleagues (20). Liver preparations (1, 19, 20, 25, 38, 46) from various animals degraded histidine with the liberation of ammonia. In contrast to the results with the whole animal shown in Fig. 1, however, there was no consumption of oxygen or production of CO<sub>2</sub>. A compound accumulated, which could be hydrolyzed by acid or alkali to ammonia, L-glutamic acid, and formic acid. This group, as well as a number of other investigators, believed that the primary enzymatic attack was on the imidazole ring (20, 38).

*Urocanic Acid Pathway.*

Meanwhile, however, evidence was accumulating for another formulation in which urocanic acid (imidazole acrylic acid) is

und bevor ich noch angefangen hatte, den Gesamtstoffwechsel des merkwürdigen Thieres zum Gegenstand eingehender Beobachtungen zu machen, ist mir der Hund eines Tages entflohen und trotz aller Nachforschungen verloren geblieben. — Ich habe seitdem die neue Substanz im Harn von 8 oder 9 anderen Hunden vergeblich gesucht und auch im menschlichen Urin nicht auffinden können.

Das entlaufene Thier — ein junger Pudel — hatte mehrere

FIG. 2. Excerpt from M. Jaffe: "Ueber einen neuen Bestandtheil des Hundeharns." *Ber. deut. chem. Ges.* 7, 1669 (1874).

formed by the non-oxidative deamination of histidine. Several investigators reported the isolation of urocanic acid from urine (32, 67, 74), but the results were variable and often could not be repeated. This is shown somewhat dramatically in Jaffe's report (32) on the first isolation of urocanic acid from the urine of a dog. The dog ran away (Fig. 2), and, even though Jaffe tried, he couldn't find him again. Attempts by Jaffe to isolate urocanic acid from other



dogs were unsuccessful. In 1912 Hunter (31) showed the structure of urocanic acid to be imidazole acrylic acid, and the probable relationship to histidine became obvious. In 1917 Raistrick (51) demonstrated the conversion of histidine to urocanic acid by bacteria, and in 1922 Kotake and Konishi (35) isolated urocanic acid from dog urine after histidine administration. Despite confirmation of these results by some investigators they could not be repeated consistently, and consequently the status of urocanic acid as a normal intermediate was questioned (16, 17, 20, 34, 38). In addition, the rapid excretion of administered urocanic acid (15, 17, 20) was considered inconsistent with its postulated role as an intermediate, particularly since there was only a small excretion of histidine or urocanic acid after histidine administration; similarly, urocanic acid did not exhibit the glycogenic properties of histidine (15).

Beginning in 1939, however, Sera and a number of other investigators in Japan (4, 48, 64-66, 85) began their extensive studies on histidine breakdown by liver. Using partially purified liver preparations, they were able to convert histidine in good yield to urocanic acid; these results were subsequently confirmed in a number of laboratories (24, 26, 49, 50). However, because of the variable results in vivo and the inability of many investigators to demonstrate urocanic acid as an intermediate in the degradation of histidine by crude liver homogenates (20, 46), the urocanic pathway was not generally accepted. The enzymatic results of Sera et al. were explained as due to a minor pathway which showed up under the conditions used.

### *Isotope Experiments.*

When Hayaishi and I became interested in this problem we investigated histidine degradation by histidine-adapted *Pseudomonas fluorescens* cells (77). Extracts of these cells stoichiometrically converted histidine to ammonia, formic acid, and L-glutamic acid (Fig. 3). This permitted us to evaluate two possible formulations of histidine breakdown. In the formulation involving a primary cleavage of the imidazole ring, the  $\alpha$ -amino group of the glutamic acid is derived from the  $\alpha$ -amino group of histidine (Fig. 4A). On the



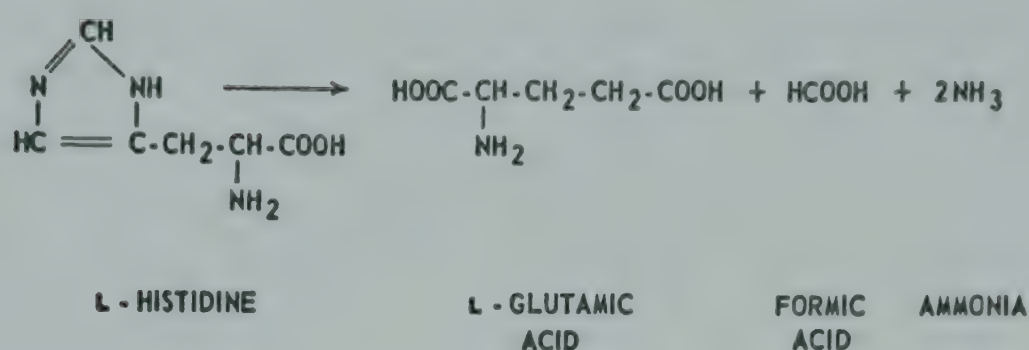


FIG. 3. Stoichiometric degradation of L-histidine to L-glutamic acid, formic acid, and ammonia by extracts of histidine-adapted *Pseudomonas fluorescens* cells.

other hand, in the formulation involving a primary deamination of the  $\alpha$ -amino group of histidine with the formation of urocanic acid as an intermediate, the  $\alpha$ -amino group of the glutamic acid is derived from the  $\gamma$ -nitrogen of histidine (Fig. 4B).

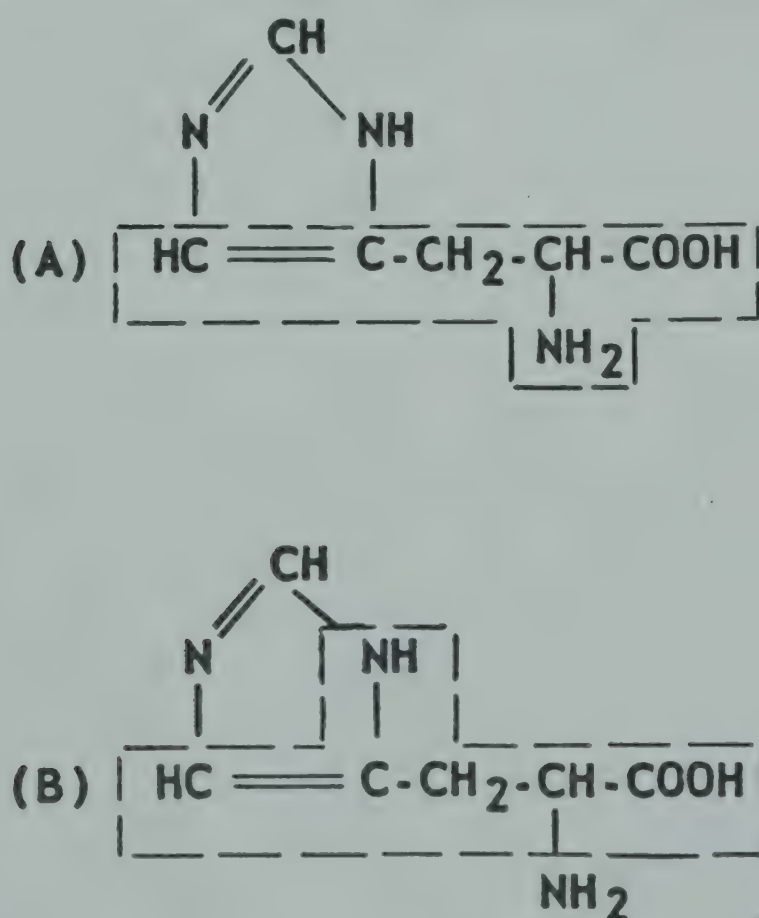


FIG. 4. The dotted lines represent the origin of the glutamic acid which is formed during the degradation of histidine (A) if there were a primary cleavage of the imidazole ring, or (B) if the urocanic acid pathway occurred.

$\text{N}^{15}$ -histidine, labeled in the  $\alpha$ - or  $\gamma$ -nitrogens, was degraded by *Pseudomonas fluorescens* extracts, and both the ammonia and glutamic acid were isolated (78). The data presented in Fig. 5 demonstrate that the  $\alpha$ -amino group of the glutamic acid came from

the  $\gamma$ -nitrogen of the histidine; the ammonia came from the  $\alpha$ -amino group and the  $\delta$ -nitrogen of the histidine. Thus, these data support formulation B. Trapping experiments, in which unlabeled urocanic acid was added to an incubation mixture of  $C^{14}$ -histidine and *Pseudomonas* extract, showed that the isotope was trapped in the urocanic acid, and thus confirmed the role of urocanic acid as an intermediate.

### DEGRADATION OF HISTIDINE LABELED WITH $N^{15}$ IN $\alpha$ - OR $\gamma$ -NITROGEN

INITIAL		FINAL			
L - HISTIDINE		L - GLUTAMIC ACID		AMMONIA	
Atom % Excess		Atom % Excess		Atom % Excess	
$\alpha$ Amino	$\gamma$ N	Found	Calculated	Found	Calculated
0.66	0.013	0.025	0.013	0.38	0.36
0.003	0.34	0.31	0.34	0.02	0.002

FIG. 5. Degradation of histidine labeled with  $N^{15}$  in  $\alpha$ - or  $\gamma$  nitrogen by cell-free extracts of *P. fluorescens*. The  $\gamma$ -N labeled histidine was kindly supplied by Dr. Charles Tesar (87). The calculated values are based on the urocanic acid formulation; the calculations for the experiment with  $\alpha$ -amino labeled histidine include the .013 and .058 atom per cent excess present in the  $\gamma$  and  $\delta$  nitrogens respectively.

The formic acid was shown to be derived from the C-2 position of the imidazole ring. These isotope results are summarized in Fig. 6. Similar trapping experiments were carried out with crude unfraktionated liver homogenates, and demonstrated that in this system also urocanic acid was an intermediate in histidine degradation (42).

These data, together with similar data obtained in the experiments in vitro and in vivo of Abrams and Borsook (2), Fournier and Bouthillier (22), Wolf (98, 99), and others (79), support the urocanic acid pathway. Although all of the older discrepancies have not been



resolved, it seems likely that the inability to obtain urocanic acid in crude liver systems was due to the presence of active enzymes in these preparations which degraded the urocanic acid as soon as it was formed. The difficulty in consistently demonstrating urocanic

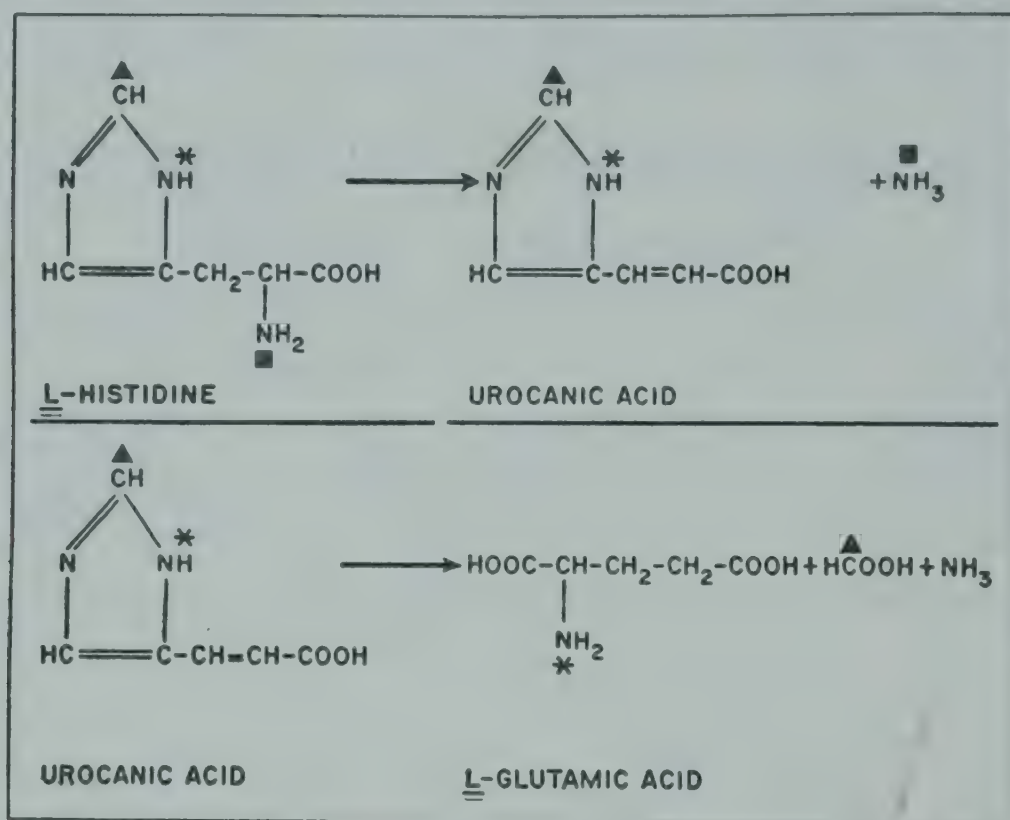


FIG. 6. Summary of N<sup>15</sup> and C<sup>14</sup> studies on histidine degradation by histidine-adapted *Pseudomonas fluorescens* extracts.

acid in the urine after histidine administration may have been due to the relative insensitivity of the isolation procedures available, while the difference in urocanic acid excretion after histidine or urocanic administration may possibly be attributed to such factors as differences in renal excretion and cell permeability.

### ENZYMATIC STUDIES

*Histidase.* Histidase is the term introduced by Edlbacher (19) to describe the enzyme preparation which degraded histidine in his experiments. Since it now seems likely that this preparation included a complex of enzymes carrying out a number of steps in the degradation, the term is now restricted to the enzyme catalyzing the conversion of histidine to urocanic acid. This enzyme has also been referred to as "desaminohistidase," "histidine  $\alpha$ -deaminase," "histidine desaminase," and "histidine deaminase" by various investigators.

I shall begin a discussion of histidase by presenting the absorption spectrum of urocanic acid (26, 42), since this has been very useful in our studies. As shown in Fig. 7, the absorption spectrum of urocanic acid has a maximum at 277 m $\mu$  (pH 7.4-11), with a molar extinction coefficient of 18,800 (42). Since histidine has essentially

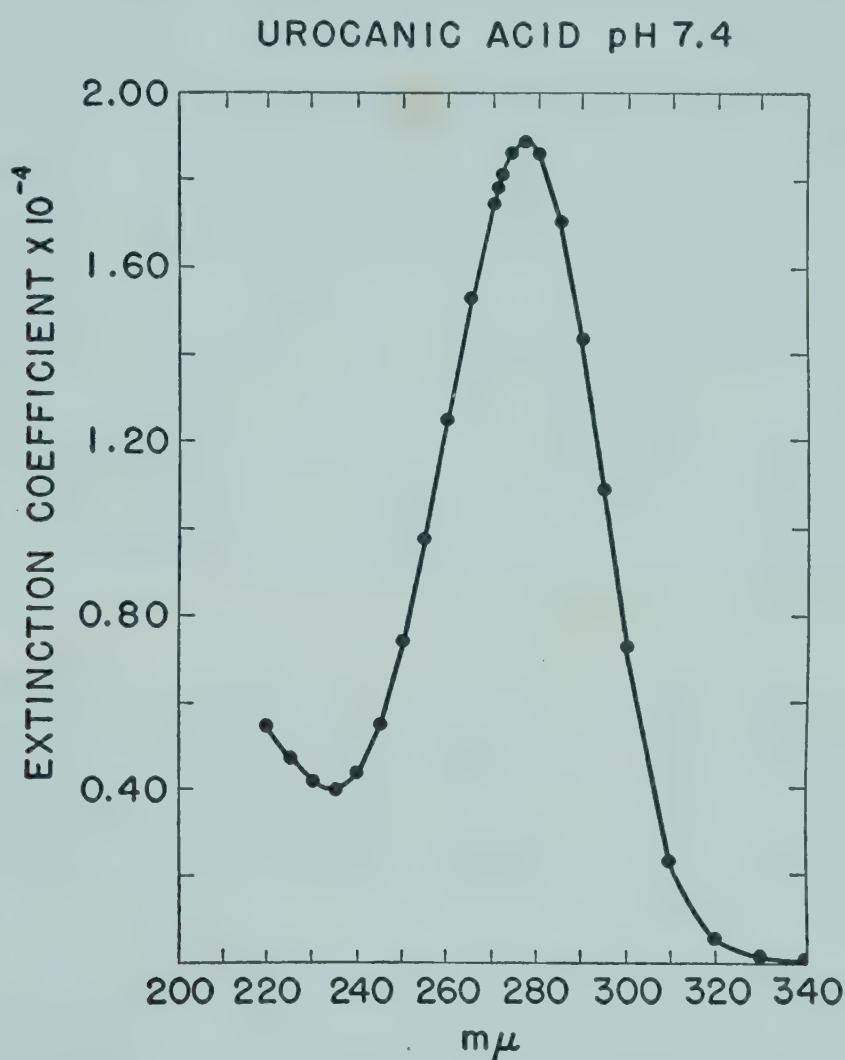


FIG. 7. Ultraviolet absorption spectrum (molar extinction coefficients) of sodium urocanate. Essentially the same spectrum is obtained from pH 7.4-11.

This absorption spectrum and the effect of pH have been presented in reference (42). Different spectra are obtained for urocanic acid hydrochloride, a zwitter-ion form (pH 4.6), and sodium urocanate; the spectra of mixtures of these forms agree well with that calculated from the respective dissociation constants (pK 3.5 and 5.8). A fourth absorption curve is found in 6.5 N KOH and has been attributed to the dissociation (pK 13) of a proton from the imidazole ring.

no absorption at 277 m $\mu$ , histidase activity can be followed easily and directly in the spectrophotometer by measuring the appearance of the absorption at this wave-length (at pH 9.3). Histidase can, of course, also be followed with ammonia or histidine determinations (19, 20, 46, 66), although those methods are less convenient and accurate as well as less specific than the spectrophotometric method.



Histidase is found in the liver of various animals, as well as in a number of bacterial sources. The enzyme is specific for L-histidine, and has been purified about 100-fold from both liver and *Pseudomonas* (20, 42, 66, 84, 85). The conversion of histidine to urocanic acid by histidase is essentially irreversible (42), and has been used for the large-scale preparation of urocanic acid (43, 66, 84, 85). The cofactor requirements are not entirely clear. Kumagai (37) has demonstrated a glutathione requirement for liver histidase; in our experiments (42) a similar requirement has been shown for *Pseudomonas* histidase, although this can be satisfied by thioglycolate or  $H_2S$ . Cysteine, on the other hand, inhibits. Both liver (9, 10, 42) and *Pseudomonas* histidase (42) are inhibited by ethylenediamine tetraacetate. Suda (72) has reported a requirement for mercurous ions, and Matsuda et al. (40) have claimed a folic acid requirement. Liver histidase shows a marked, but unexplained, stimulation by pyrophosphate (42).

*Urocanase.* The enzyme degrading urocanic acid (urocanase) is conveniently studied by measuring the disappearance of the urocanic acid absorption at 277 m $\mu$ . Only preliminary studies, however, (42, 66, 89), have been carried out on the purification of this enzyme or its characteristics.

The degradation of histidine or urocanic acid by liver preparations or by partially purified *Pseudomonas* preparations results in the accumulation of an intermediate product which releases ammonia, formic acid, and L-glutamic acid upon alkaline hydrolysis. Crystallization of this intermediate has been reported by Sera and Aihara (65), Oyamada (48), Borek and Waelsch (9, 10), and Tabor and Mehler (82),<sup>1</sup> although it is not certain that the same compound was crystallized in the various laboratories. There has been considerable disagreement on the structure of this intermediate, and some of the structures which have been postulated are: formyl-L-glutamine (20), formyl-DL-isoglutamine (48, 66), and formamido-L-glutamic acid (9, 10, 82, 92).

<sup>1</sup> A similar compound has been isolated from the urine of folic-acid deficient rats by Silverman et al. (68), and will be discussed in greater detail below.



An important contribution has recently been made by Borek and Waelsch (9, 10), who have reported the presence of one basic and two acidic groups in their compound. They therefore presented L-formamidinoglutamic acid (formimino-L-glutamic acid, formamido-L-glutamic acid) as the most likely structure. Similar titration data have subsequently also been reported by Tabor and Mehler (82) for the intermediate compound isolated in their laboratory. Further support for the formamido-L-glutamic acid structure has been obtained by the recent syntheses of Miller and Waelsch (44) and of Seegmiller et al. (63). Thus, the pathway postulated at present for the degradation of histidine (10, 73, 82), involves the deamination of histidine to form urocanic acid, the addition of one molecule of water, and rearrangement to form imidazolone propionic acid, and the addition of another molecule of water to form formamido-L-glutamic acid. The postulated imidazolone propionic acid is still an unknown compound.

*Degradation of Formamido-L-Glutamic Acid.* The further degradation of histidine beyond formamido-L-glutamic acid varies in different preparations. In liver homogenates (10, 20, 66, 82), for example, there is very little degradation beyond this stage, even though the whole animal rapidly degrades histidine to  $\text{CO}_2$ . Although the formation of isoglutamine and glutamic acid has been described during the degradation of histidine and urocanic acid (2, 22, 48, 66, 85), considerable caution has to be exercised in evaluating these reports, since formamidoglutamic acid can easily undergo non-enzymatic degradation (10, 44, 63, 68, 82). Recently, however, Abrams and Knudson (unpub.) have demonstrated the conversion of  $\text{C}^{14}$ -formamidoglutamic acid to  $\text{C}^{14}$ -glutamic acid in rat liver extracts. A non-enzymatic degradation was ruled out by control experiments with boiled enzyme. The conversion of histidine to glutamic acid in vivo has been reported both by Abrams and Borsook (2) and by Wolf (98). Following the administration in vivo of  $\text{C}^{14}$ -histidine,  $\text{C}^{14}$ -glutamic acid was isolated from a non-protein liver fraction (Abrams and Borsook) and from a protein hydrolysate (Wolf). No explanation is available at present for the



sluggish nature of the later steps of histidine degradation by liver systems in vitro, although the possible lack of a suitable one-carbon transfer system has been suggested (10, 82).

In contrast to the results with liver homogenates, *Pseudomonas* extracts rapidly convert histidine, urocanic acid, and formamido-glutamic acid (10, 71, 82) to stoichiometric quantities of L-glutamic acid, formic acid, and ammonia. Recently, evidence has been presented by Suda et al. (73) and from our laboratory (82) that formyl-L-glutamic acid is the immediate precursor of the glutamic acid in this degradation by *Pseudomonas* extracts. The conclusions of Suda

SUBSTRATE 20 $\mu$ M	$\mu$ M OF GLUTAMIC ACID PRODUCED BY EXTRACTS OF	
	NORMAL CELLS	HISTIDINE- ADAPTED CELLS
HISTIDINE	0.0	10.0
FORMYLGLUTAMIC ACID	0.0	16.0

FIG. 8. The formation of glutamic acid from histidine and from formylglutamic acid in extracts from non-adapted and histidine-adapted cells. Data taken from Suda et al. (73).

et al. were based on simultaneous successive adaptation experiments (73) (Fig. 8). The isolation of formyl-L-glutamic acid as an intermediate was reported by Tabor and Mehler (82). A summary of the overall pathway in *Pseudomonas* extracts is presented in Fig. 9. In the intact cell the glutamic and formic acids are then degraded to  $\text{CO}_2$  or used for synthetic purposes.

Only preliminary work has been carried out on the enzymes in *Pseudomonas* which degrade formamido-L-glutamic and formyl-L-glutamic acids. Although very little purification has been achieved, the two activities can be easily separated by differential inhibition by  $\text{H}_2\text{S}$  and by aging. The enzyme which degrades formamido-L-glutamic acid is protected by  $\text{H}_2\text{S}$ , while the enzyme which hydrolyzes

formyl-L-glutamic acid (glutamic formylase) is inhibited by  $\text{H}_2\text{S}$ . The activity of glutamic formylase is also lost on dialysis or aging, and can be restored by the addition of ferrous ions. Glutamic formylase appears to be rather specific for formylglutamic acid, as there is little or no splitting of acetylglutamic acid or a variety of other formylated amino acids (82).

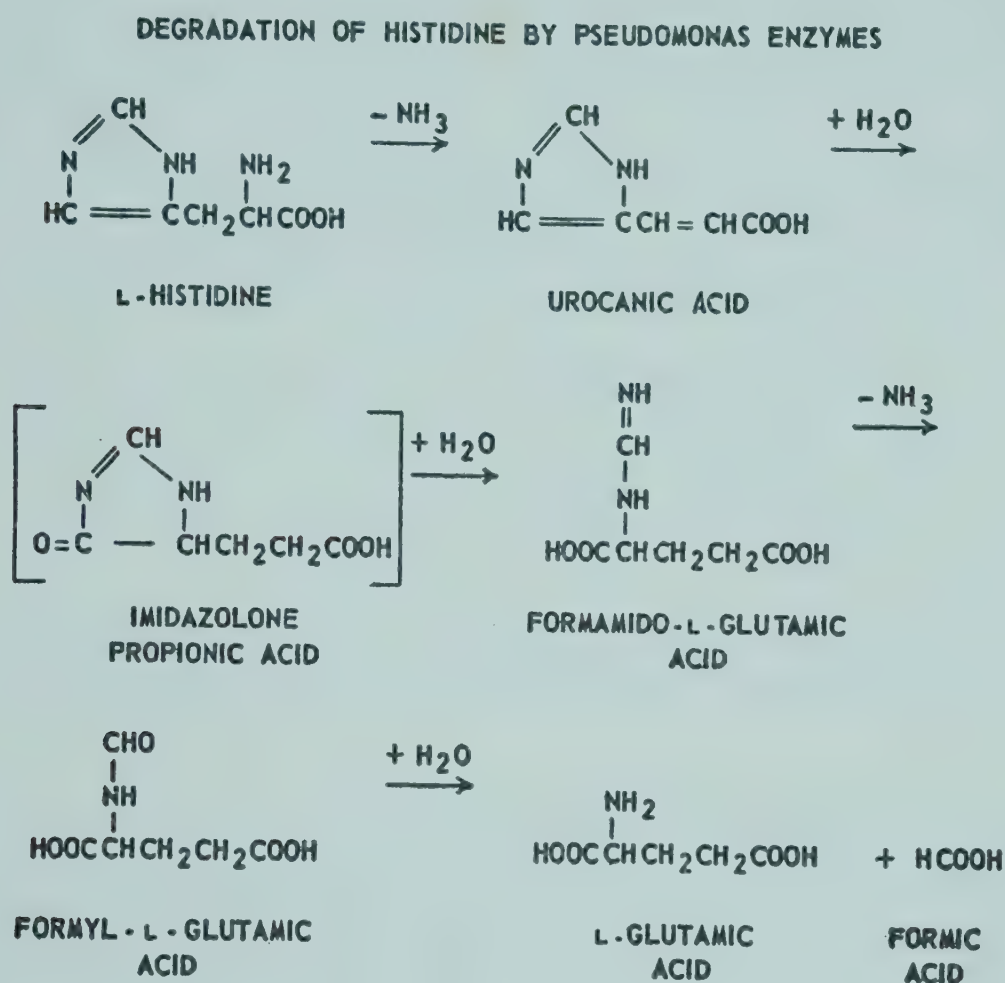


FIG. 9. Degradative pathway in *Pseudomonas* (73, 82). The pathway up to formamido-L-glutamic acid has also been presented for the data obtained with liver preparations (10).

Still another pathway is indicated by the isolation of formamide in the recent experiments of Magasanik with *Aerobacter aerogenes* (39) and of Wachsmann and Barker (91) with *Clostridium tetanomorphum*.

#### RELATIONSHIP OF HISTIDINE DEGRADATION AND FOLIC ACID

A particularly interesting aspect of the problem of histidine degradation is the relationship of histidine degradation to folic acid and one-carbon metabolism. Some years ago Bakerman, Silverman,



and Daft (6, 68) reported the accumulation of a labile glutamic derivative in the urine of folic-deficient rats, but not in the urine of normal rats. This compound was crystallized as the barium salt (68), and has been identified by Silverman, Tabor, Mehler, Daft, and Bauer (63, 79) with the compound (formamido-L-glutamic acid) which had been isolated as a degradation product of histidine by liver preparations (above). Folic acid-deficient rats were then fed  $N^{15}$ -labeled histidine; the glutamic derivative was isolated from

	MILLI-MOLES	ATOM PERCENT EXCESS $N^{15}$
L - HISTIDINE * FED	10.6	4.8 (IN $\gamma$ N)
GLUTAMIC DERIVATIVE EXCRETED	4.6	2.74 (IN $\alpha$ N)

\*  $N^{15}$  IN  $\gamma$  POSITION

FIG. 10. Excretion of  $N^{15}$ -labeled glutamic derivative (formamido-L-glutamic acid) in the urine following the administration of  $N^{15}$ -labeled histidine to folic-acid-deficient rats (79).

the urine, and shown to contain most of the isotope (Fig. 10). These data indicate that the urinary compound was derived from histidine. Furthermore, since the glutamic moiety of this compound still contained the  $N^{15}$  that was in the  $\gamma$ -position of the histidine, this experiment offers further indication for the occurrence in vivo of the urocanic acid pathway.

The relationship of histidine degradation to one-carbon metabolism has also been shown by Sprinson and Rittenberg (70), Soucy and Bouthillier (69), Reid, Landefield, and Simpson (52, 53), and Toporek, Miller, and Bale (88), who have demonstrated that carbon-2 of the imidazole ring is incorporated in vivo into serine and other formate acceptors. In preliminary experiments Mehler and I have recently been able to demonstrate this fixation reaction in vitro. Labeled histidine was converted enzymatically to labeled urocanic acid and thence to labeled formamido-L-glutamic acid. This was incubated with a pigeon liver homogenate, and about 1 per cent of the isotope was found to be incorporated into an inosinic acid frac-



tion. Further work is in progress to decide whether this incorporation of the label from histidine occurs via a preliminary conversion to formic acid (93) or by some other formate-transfer reaction.

#### DECARBOXYLATION OF HISTIDINE AND HISTAMINE METABOLISM

There is insufficient time to discuss in detail most of the other metabolic relationships of histidine, such as the incorporation of histidine into proteins (12), the conversion of histidine to imidazole pyruvic acid by oxidative deamination (8, 36) or transamination (14), or the isolation of imidazole lactic and imidazole propionic acids from bacterial cultures (3, 28, 34). Histidine can be converted to carnosine ( $\beta$ -alanylhistidine) (97) and presumably to anserine. The pathway via histamine is of particular interest because of its possible physiological and pharmacological significance, even though quantitatively it represents only a small proportion of the histidine degraded in animals. Therefore this pathway will be outlined briefly, even though there is insufficient time to review adequately the extensive literature involved. (This literature has recently been reviewed more extensively in ref. 83).

*Histidine decarboxylase.* Histidine decarboxylase has been studied in various microorganisms, particularly in *Clostridium welchii*, *Escherichia coli*, and *Lactobacillus* sp. (21, 23, 27, 55, 58, 86). The bacterial enzyme has been partially purified, but, as Meister has already stated, so far no cofactor has been clearly demonstrated. The enzyme from animal tissues (58) was described by Werle (94-95) and by Holtz (29). Its activity, however, is very low, and pharmacological assays were used to follow the appearance of histamine. Here again, even though there have been some reports of a pyridoxal phosphate (30, 96) effect, the data are not unequivocal. The formation of histamine has recently been demonstrated in vivo by the  $C^{14}$  experiments of Schayer (60).

*Histaminase (Diamine Oxidase).* Histamine may be acetylated (5, 45, 54, 56, 75, 81, 90), or it may be degraded by the enzyme histaminase (7). This enzyme is probably the same as Zeller's diamine oxidase (100, 101), although there is still some question



on this point (33, 57). The enzyme has been studied by a number of investigators, including Best et al., Gebauer-Fuelnegg, Kapeller-Adler, Laskowski, Swedin, Stephenson, Zeller, Blaschko, Tabor, and others (see ref. 83 and 101 for references). The first product in the oxidation of histamine by this enzyme is presumably imidazole acetaldehyde, although this compound has never been adequately characterized as an intermediate. The product of the action of diamine oxidase on histamine, however, can be converted (76) by aldehyde dehydrogenase and diphosphopyridine nucleotide or by xanthine oxidase to imidazole acetic acid, which has been isolated and identified (Fig. 11).

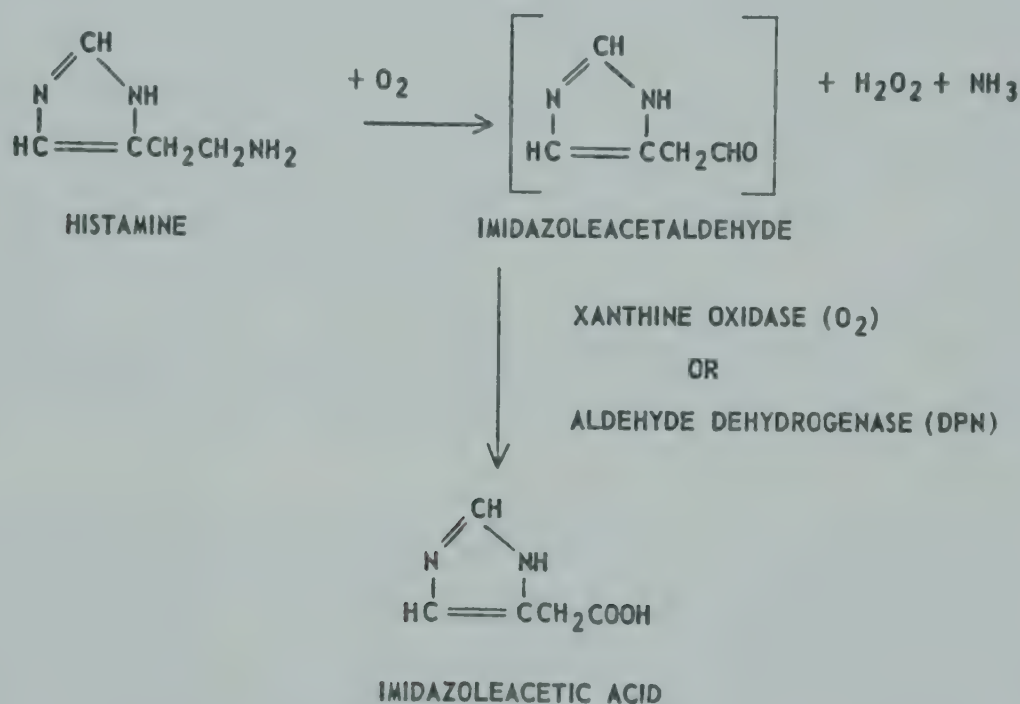


FIG. 11. Enzymatic oxidation of histamine to imidazole acetic acid.

The conversion of histamine to imidazole acetic acid has also been confirmed *in vivo* by the isolation of imidazole acetic acid from the urine of rats after histamine administration (13, 41, 80), although Schayer has recently presented evidence that another pathway is also operative in several species (59, 61, 62). Studies are in progress on the further metabolism of imidazole acetic acid, both in animals and in *Pseudomonas*. These experiments are being carried out in collaboration with Dr. Hayaishi, who will present these results during the discussion.

The final figure represents a summary of some of the metabolic interrelationships of histidine (Fig. 12).

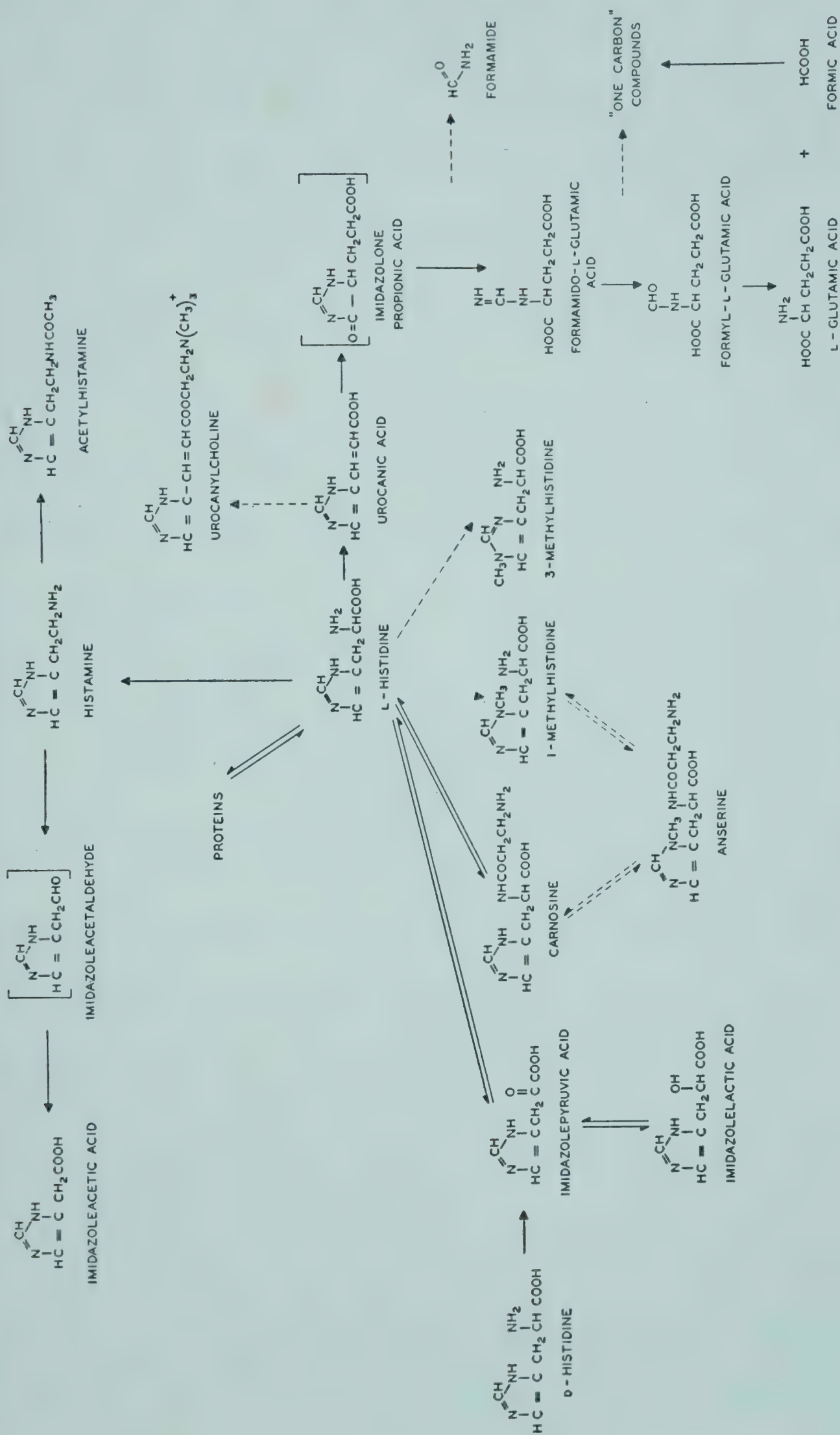


FIG. 12. Metabolic interrelations of histidine.

(Abstracted from Fig. 1 in ref. 83 with the permission of *Pharmacological Reviews* and The Williams and Wilkins Company.)



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# ENZYMATIC STUDIES ON THE METABOLISM OF IMIDAZOLEACETIC ACID \*

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DURING THE PAST few years, the metabolic pathway from histidine to glutamic acid by way of urocanic acid has been a subject of extensive investigation by several groups of workers. The brilliant outcome of these intensive studies has been presented by Tabor, who has summarized our present knowledge of this phase of histidine metabolism. Tabor's paper was of particular interest to me because the role of urocanic acid in histidine metabolism was first proposed about 15 years ago by Drs. Sera and Kotake, who were my teachers back in Osaka University.

Another metabolic pathway of histidine, which may be less significant in a quantitative sense but physiologically more important, is the formation of histamine by histidine decarboxylase, a path which is found in mammals as well as in microorganisms. Histidine is further metabolized to imidazole acetic acid (ImAA) by way of imidazole acetaldehyde, as shown in scheme A (Fig. 1). The topic I will discuss today is concerned with the further metabolism of imidazole acetic acid in mammals and bacteria, which we have been studying in collaboration with Dr. Tabor and his associates at the National Institutes of Health.

The overall reaction in ImAA-adapted *Pseudomonas* extracts is shown in scheme B (Fig. 1). One mole of DPNH and one mole of oxygen are consumed per mole of imidazole acetic acid, and one mole of formylaspartic acid and one mole of ammonia are

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produced. Because of the adaptive nature of the enzyme, *Pseudomonas fluorescens* strain No. 6 was grown for 16 hours at 30° C. with mechanical stirring in a medium containing 0.1% imidazole acetic acid, 0.1% Difco yeast extract, 0.15%  $K_2HPO_4$ , and 0.05%  $KH_2PO_4$ , and 0.02%  $MgSO_4 \cdot 7H_2O$ . Cell-free extracts were prepared by grinding the washed cells with alumina (Alcoa A-301), extracting with 10 parts of 0.02 M. potassium phosphate buffer, pH 7.0, and centrifuging at  $25,000 \times g$  for 30 minutes. The activity was deter-

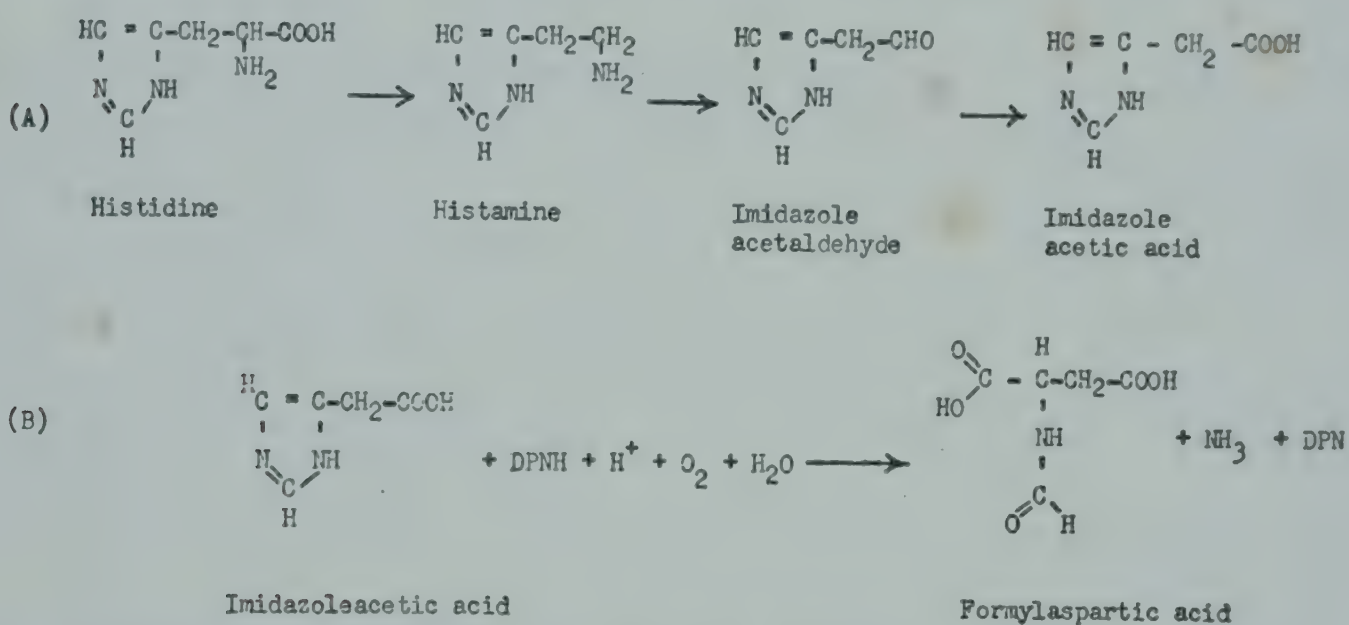


FIG. 1. Metabolic pathway of histidine by way of histamine and imidazole acetic acid.

mined by the rate of oxidation of DPNH as measured by the decrease of absorption at 340 m $\mu$  in the presence of imidazole acetic acid and Tris buffer (pH 9.2, 0.1 M.). Using this assay system, the enzyme was purified about 200-fold by protamine treatment and repeated adsorption and elution on and from calcium phosphate gel and alumina C $\gamma$  gel. The final preparation was completely free of DPNH oxidase, which was present in the crude extract.

When the reaction was carried out under strictly anaerobic conditions, no reaction was detectable either by disappearance of DPNH or by colorimetric determination of imidazole acetic acid (1). When the reaction was run in a Beckman cuvette, which is attached to a Thunberg tube, and the atmosphere was completely replaced by oxygen-free nitrogen, the addition of ImAA did not cause any detectable disappearance of DPNH, as shown in Fig. 2. The ion exchange chromatogram of such an anaerobic incubation was indis-

tinguishable from that of ImAA which will be shown in Fig. 3 (A). Upon addition of a small amount of air, DPNH was immediately oxidized. If ImAA was omitted from the incubation mixture, no oxidation of DPNH was observed.

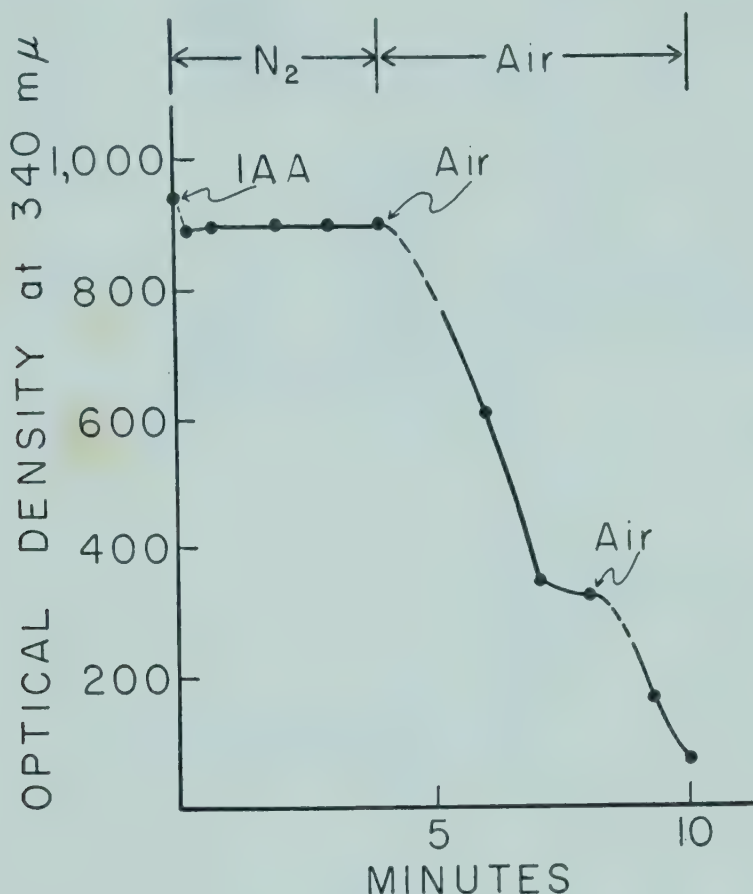


FIG. 2. Effect of oxygen on the DPNH oxidation.

Incubation mixture (3.0 ml.) contains 300  $\mu$ M. of Tris buffer (pH 9.4), 0.4  $\mu$ M. of DPNH,\* 10  $\mu$ M. of ImAA, and 0.2 ml. of purified enzyme preparation. When ImAA is omitted, no reaction was observed.

\* Prepared according to Ohlmeyer, *Biochem. Z.* 297, 66 (1938).

When the incubation was carried out in an ordinary Beckman cuvette, with occasional shaking to permit mild aeration in the presence of a limited amount of DPNH, the reaction can be followed by the disappearance of DPNH. ImAA disappeared as shown in Fig. 3 (B), and at least two products were detectable on the chromatogram. When the incubation mixture was vigorously aerated, practically all the ImAA present was converted to a single compound, which was tentatively identified as formylaspartic acid.

Under these conditions, one mole each of oxygen and DPNH was consumed, and one mole each of ammonia and a bound form of



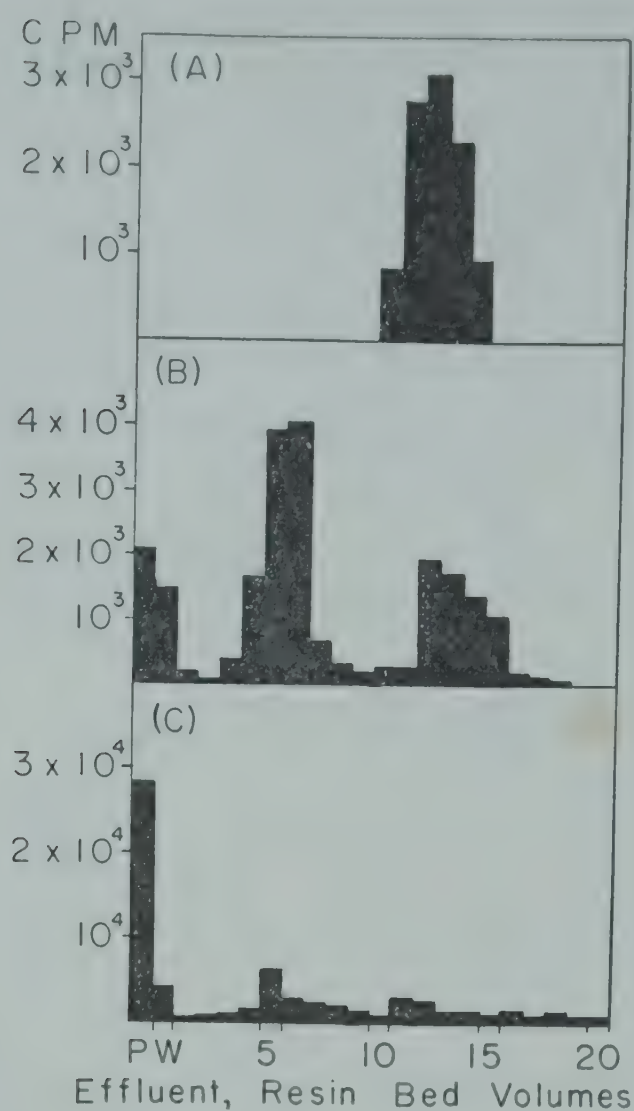


FIG. 3. Ion exchange chromatogram.

(A) One  $\mu$ mole of carboxyl-labeled ImAA (18,000 c.p.m.) was adsorbed on Dowex-50 ( $K^+$  form) column, 3.0 cm.  $\times$  1 sq. cm., and eluted with 0.5 M. HCl.

(B) The reaction mixture (6 ml.) containing 300  $\mu$ moles of Tris buffer, pH 9.6, 4  $\mu$ moles of ImAA (36,000 c.p.m.), 2  $\mu$ moles of DPNH, 0.6 ml. of partially purified enzyme (0.24 mg. protein) was incubated for 30 minutes at 27° C. The reaction was followed by the disappearance of absorption at 340 m $\mu$ . After the reaction went almost to completion, the incubation mixture was deproteinized with 2% perchloric acid, and the supernatant was chromatographed after perchlorate was removed as potassium salt on a Dowex-50 column, 3.0  $\times$  1 sq. cm., and eluted with 0.5 M. HCl.

(C) The reaction mixture (40 ml.) containing 500  $\mu$ moles of ImAA (45,000 c.p.m.), 8 millimoles of glucose, 2.0 ml. of glucose dehydrogenase, 4 millimoles of Tris buffer, pH 9.6, 4  $\mu$ moles of DPN, and 8.0 ml. of partially purified *Pseudomonas* enzyme (2.4 mg. of protein) were incubated for 2 hours at 30° C. with vigorous aeration. The incubation mixture was deproteinized with 2% perchloric acid, and the supernatant was chromatographed on Dowex-50 (K-form) column, 5.0  $\times$  1. sq. cm., and eluted with 0.5 M. HCl.

aspartic acid was produced. No CO<sub>2</sub> evolution was observed (Table 1). The bound aspartic acid can be converted to free aspartic acid after acid hydrolysis (1.0 N H<sub>2</sub>SO<sub>4</sub>, 100° C. 30 minutes) and was

TABLE 1

Assay	Experimental $\mu M.$	Control * $\mu M.$
ImAA disappearance	10.0	0
Acid labile aspartic acid	9.4	0
Ammonia	9.8	0
Oxygen consumption	9.6	0.14
CO <sub>2</sub> production	0.6	0.4

Incubation mixture (1.2 ml.) contained 10  $\mu M.$  of ImAA, 100  $\mu M.$  of Tris buffer, pH 9.4, 200  $\mu M.$  glucose, 0.05 ml. of glucose dehydrogenase [H. J. Strecker and S. Korkes, *J. Biol. Chem.* 196, 769 (1952)], 0.1  $\mu M.$  of DPN and 0.5 ml. of crude extract. Incubation 70 minutes at 30° C.

\* Incubation mixture identical with the experimental mixture except for the omission of glucose dehydrogenase and glucose.

determined quantitatively by an enzymatic method (2). The isolated crystalline compound melted at 132-135° C. (uncorrected), as did the authentic specimen \* and a mixture of the two. The behavior of the product on paper chromatography with two solvent systems further established the identity with formylaspartic acid (Table 2).

TABLE 2

	Solvent 1 *	Solvent 2 **
Aspartic acid	0.18	0.34 ~ 0.37
Formylaspartic acid	0.52	0.69 ~ 0.72
Sample	0.52	0.68 ~ 0.72
Samples after acid hydrolysis	0.18	0.34 ~ 0.37

\* Solvent 1. Ethanol, 77; H<sub>2</sub>O, 23.

\*\* Solvent 2. Tertiary butanol, 70; formic acid, 15; water, 15.

The behavior of the ImAA-adapted and non-adapted cells towards formylaspartic acid was studied with a conventional manometric technique. As shown in Fig. 4, the cells adapted to ImAA can oxidize formylaspartic acid as well as aspartic acid without a lag period, whereas the cells grown in tryptose broth show a distinct adaptive lag with all three substrates. This result seems to indicate that both

\* Kindly furnished by Dr. A. H. Mehler.



formylaspartic and aspartic acids are actually intermediate metabolites of imidazole acetic acid, and are not artifacts which are produced during the isolation procedure.

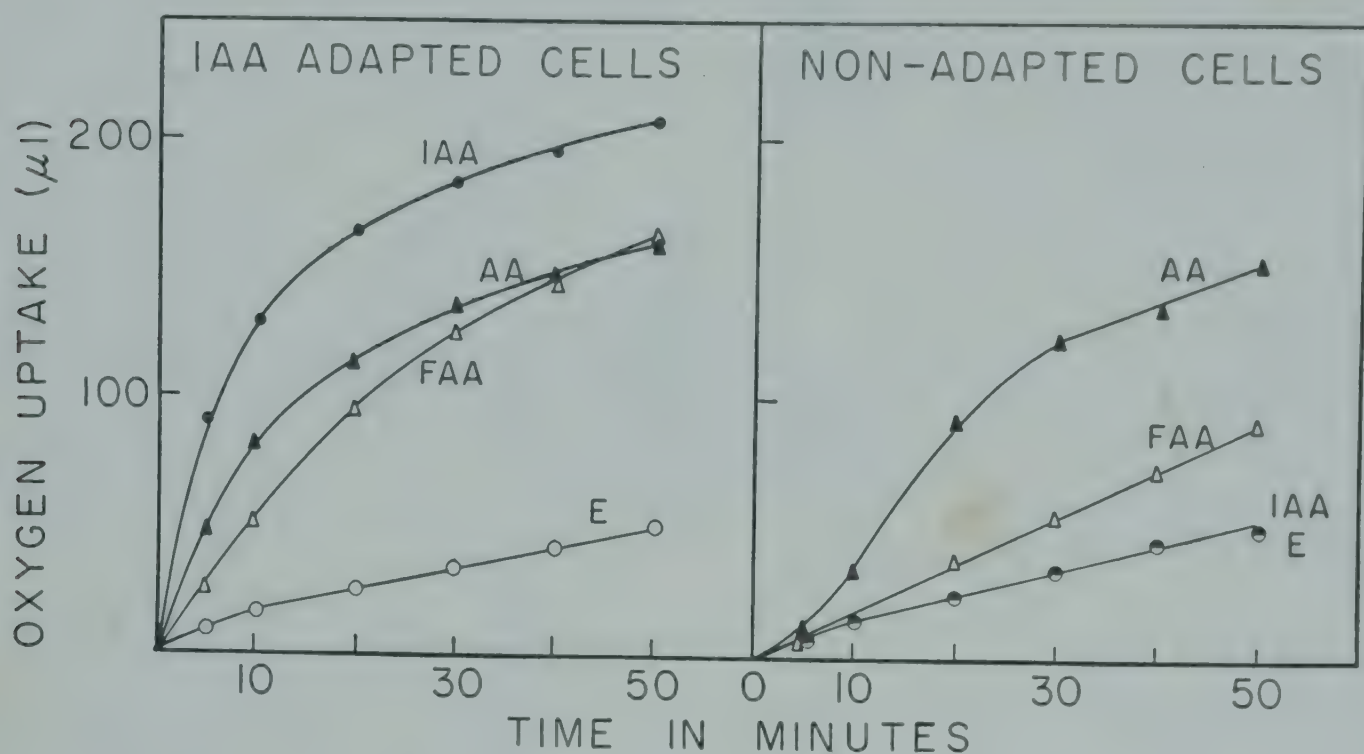


FIG. 4. Oxygen uptake by cells grown in the presence of imidazole acetic acid and in tryptose broth.

Each Warburg vessel contained  $4 \mu\text{M}$ . of substrate and about 1 mg. (dry weight) of cell material in a total volume of 2.0 ml. of 0.02 M. phosphate buffer, pH 7.0; 0.2 ml. of 10% KOH was in the center well. Temperature  $31^\circ \text{C}$ .

The mechanism of the reaction and the nature of the intermediates are under investigation. In most experiments glucose and glucose dehydrogenase were present to regenerate a catalytic amount of DPNH. When glucose dehydrogenase was replaced by glucose oxidase (notatin), obtained from *Penicillium notatum*, ImAA did not disappear to any detectable extent. This observation, together with the absence of DPNH oxidase in the purified preparation, seems to exclude the possibility that peroxidative mechanism is involved in this reaction.

The enzyme appears to be relatively specific, since the rate of oxidation of DPNH with imidazole propionic acid was less than 5 per cent of that with imidazole acetic acid, and no reaction was observed with histamine, histidine, imidazole, or imidazole lactic acid.

In order to investigate the metabolism of imidazole acetic acid in mammals, carboxyl-labeled ImAA was administered intraperitoneally to rats. Essentially no radioactivity was found in the expired  $\text{CO}_2$ , in agreement with the result of Bouthillier and L  veill   (3). A crystalline compound was isolated from the urine which behaved differently from ImAA on both paper and Dowex-50 chromatography, as well as in melting point and elementary analysis. This compound has not been completely characterized yet, but it appears to be a conjugate of ImAA with a 5- or 6-carbon carbohydrate moiety.

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# PATHWAYS OF L-HISTIDINE DEGRADATION IN MICROORGANISMS \*

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THE OBSERVATION that a histidineless mutant of *Aerobacter aerogenes* required 20 times as much histidine for full growth on *myo*-inositol as on glucose (14) led to an investigation of the histidine metabolism of this organism. It was found that histidine was degraded by adaptive enzymes whose synthesis was suppressed by glucose, but not by *myo*-inositol or other carbon compounds (6). The production of the enzyme system by the histidineless mutant growing on *myo*-inositol caused the rapid dissimilation of the histidine supplied in the medium and thus limited the growth of the organism. The present study deals with the nature of the histidine-degrading enzyme system of *A. aerogenes*<sup>1</sup> and compares the pathways of histidine dissimilation of *A. aerogenes* and of *Pseudomonas fluorescens*.

## ENZYMATIC ADAPTION TO HISTIDINE

Exposure of *A. aerogenes* to histidine causes the formation of enzymes which permit the organism to grow on histidine as the only source of carbon and of nitrogen. N-acyl-derivatives of histidine, such as acetyl-histidine or glycyl-histidine, do not induce the formation of this enzyme system, but can satisfy the histidine requirement of the histidineless mutant. It was observed that glucose-grown as well as histidine-grown cells could hydrolyze the acyl-histidines;

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<sup>1</sup> In this work the histidineless mutant H-50 was used.

however, the histidine is liberated too slowly to induce the synthesis of the histidine-degrading enzymes.

Suspensions of histidine-grown cells, in contrast to glucose-grown cells, can oxidize histidine as well as glutamic acid at a rapid rate. It is of interest that intact cells cannot degrade histidine anaerobically, but that *vacuum*-dried histidine-adapted cells convert it readily to glutamic acid under these conditions. It would seem that energy, which can be obtained by endogenous respiration, is required to transport histidine across the intact cell wall. Urocanic acid is not attacked by resting cells even in the presence of oxygen, but is rapidly converted to glutamic acid by histidine-grown *vacuum*-dried cells. Apparently, the cell wall is quite impermeable to urocanic acid.

A somewhat different behavior toward these compounds is shown by a *Pseudomonas* strain (9). Histidine-grown cell suspensions oxidize histidine, glutamic acid, and urocanic acid, and convert histidine anaerobically to glutamic acid. This organism would therefore appear to be more permeable to urocanic acid than the *Aerobacter* strain used in our experiments. Adaptation of the *Pseudomonas* strain to urocanic acid enables it to attack histidine and glutamic acid as well (9). On the other hand, adaptation to glutamic acid does not endow either one of the organisms with the ability to attack histidine or urocanic acid.

The "adaptive patterns" of both bacterial species suggest that in each case histidine is dissimilated via urocanic acid and glutamic acid.

#### DEGRADATION OF HISTIDINE BY CELL SUSPENSIONS OF *A. aerogenes*

The oxidation of histidine proceeds with the evolution of carbon dioxide. During the early stages of the degradation small amounts of urocanic acid and of glutamic acid could be demonstrated in the suspending medium, but these disappeared as the oxidation progressed (Table 1, Exps. 1 and 2). The amounts of oxygen taken up and of CO<sub>2</sub> released when the rate of oxygen uptake had returned to



endogenous levels were the same with histidine and with glutamic acid as substrates (Table 1, Exps. 3 and 4), and correspond to about one half of the theoretical values for the complete oxidation of glutamic acid to  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , and  $\text{NH}_3$ . Thus it appears that a fraction of every molecule of either histidine or glutamic acid is assimilated as material of the composition  $2.5(\text{CH}_2\text{O})$ . A similar balance of

TABLE 1  
OXIDATION OF HISTIDINE AND OF GLUTAMIC ACID BY HISTIDINE-GROWN  
CELL SUSPENSIONS OF *A. aerogenes*

Experiment Substrate Time, min.	1 Histidine 10	2 Histidine 40	3 Histidine 60	4 Glutamic Acid 110
	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$
Substrate	—5.5	—10.0	—10.0	—10.0
$\text{O}_2$			—23.0	—25.0
$\text{CO}_2$			+26.4	+26.5
Urocanic acid	+0.3	0		
Ammonia, alkali-labile	+3.8	+ 9.2	+ 9.5	+ 0.7
Formic acid, " "	+3.5	+ 8.0		
Glutamic acid, free	+1.1	0		
Ammonia, " "	+8.1	+14.3	+16.8	+ 6.8
Formic acid, " "	+0.5	+ 1.3		

Each Warburg vessel contained 0.75 ml. of resting cells suspended in 0.067M. phosphate buffer of pH 6 (optical density, measured in a Coleman spectrophotometer, model 14, at 590  $\text{m}\mu$ , about 0.1), and 0.25 ml. water containing 10  $\mu\text{moles}$  of substrate. The results are corrected for endogenous respiration. At the time indicated, the cells were removed by centrifugation, and the supernatant fluid analyzed. Urocanic acid was identified spectrophotometrically and by paper chromatography, and was determined as described by Mehler and Tabor (8). Glutamic acid was determined colorimetrically after paper chromatography (1). Ammonia was determined by microdiffusion (3). Formic acid was distilled and estimated as described by Grant (4).

dissimilation and assimilation was noted when other carbon compounds were degraded by *A. aerogenes* (7). In addition, about 0.3 molecules of  $\text{NH}_3$  appear to be assimilated for every molecule of histidine or glutamic acid oxidized. The remainder of the histidine accumulates in the suspending medium as ammonia and an equimolar mixture of alkali-labile ammonia and alkali-labile formic acid (Table 1).

The results of these experiments indicate that histidine is converted via urocanic acid to formamide and glutamic acid. The latter is then partly oxidized to  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , and  $\text{NH}_3$ , and partly assimilated.

# DEGRADATION OF UROCANIC ACID BY CELL-FREE EXTRACTS OF *A. aerogenes* AND OF *P. fluorescens*

Urocanic acid is degraded by extracts of histidine-adapted *A. aerogenes* and of *P. fluorescens*<sup>2</sup> (13) more rapidly than histidine. The crude enzyme systems prepared from either organism, when used in low concentrations, degraded urocanic acid without the production of glutamic acid or of ammonia; the product of the reaction could be hydrolyzed by alkali to an equimolar mixture of glutamic acid and of ammonia, equivalent to about 75 per cent of the urocanic acid that had disappeared (Table 2, Exps. 1 and 2).

TABLE 2

DEGRADATION OF UROCANIC ACID BY CELL-FREE EXTRACTS OF HISTIDINE-GROWN  
*A. aerogenes* AND *P. fluorescens*

Experiment	1	2	3	4
Enzyme, source	<i>A. aerogenes</i>	<i>P. fluorescens</i>	<i>A. aerogenes</i>	<i>P. fluorescens</i>
" ml.	0.1	0.1	0.6	0.6
	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$
Urocanic acid	—4.5	—8.3	— 9.7	—10.0
Glutamic acid, alkali-labile	+3.2	+6.3	+ 0.4	+ 3.2
Ammonia, " "	+4.0	+6.3	+ 8.1	+ 3.5
Formic acid, " "			+10.4	+ 2.1
Glutamic acid, free	0	trace	+10.0	+ 5.5
Ammonia, "	0	+0.5	+ 1.0	+ 6.1
Formic acid, "			0	+ 5.5

The reaction mixture consisted of 1.0 ml. 0.067 M. phosphate buffer, pH 6.3, containing the amount of enzyme indicated (0.1 ml. of the *Aerobacter* extract contained 0.6 mg. of protein), and 10  $\mu\text{M}$ . of neutralized urocanic acid. The mixtures were incubated at 28° C. and at 37° C., in the case of the *Pseudomonas* and the *Aerobacter* extracts, respectively. Changing the pH to 7.0, or increasing the temperature to 37° C., did not affect the balance of the products. The duration of experiments 1 and 2 was 75 min., and of experiments 3 and 4, 110 min.

<sup>2</sup> Strain 6, kindly supplied by Dr. Herbert Tabor.



Paper chromatography revealed the presence of a single compound producing a purple spot about one week after contact with ninhydrin. In the butanol-formic acid solvent used, this compound moved faster than glutamic acid and was identical in its  $R_F$  value and its behavior toward the ninhydrin reagent with the compound isolated by Borek and Waelsch from histidine degraded by a cat-liver preparation, and identified as  $\alpha$ -formamidino glutaric acid<sup>3</sup> (2).

The action of more concentrated extracts of the two organisms on urocanic acid led to different end-products. The *Pseudomonas* preparation produced an equimolar mixture of glutamic acid, ammonia, and formic acid; under the conditions used the reaction did not go to completion, and about one-third of the urocanic acid was accounted for as an alkali-labile glutamate derivative (Table 2, Exp. 4). The *Aerobacter* preparation degraded urocanic acid to glutamic acid and a compound hydrolyzable by alkali to ammonia and formic acid. Only traces of free ammonia were produced (Table 2, Exp. 3).

TABLE 3

DEGRADATION OF THE ALKALI-LABILE INTERMEDIATE BY CELL-FREE EXTRACTS OF HISTIDINE-GROWN *A. aerogenes* AND *P. fluorescens*

Experiment	1	2	3
Enzyme, source	<i>P. fluorescens</i>	<i>A. aerogenes</i>	<i>A. aerogenes</i>
" ml.	0.6	0.6	0.6
Substrate, source	<i>A. aerogenes</i> *	<i>P. fluorescens</i> *	Synthetic <sup>†</sup>
	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$	$\Delta \mu\text{moles}$
Glutamic acid, alkali-labile			—8.6
Ammonia, " "	—4.1	+0.2	+0.3
Glutamic acid, free	++ #	+++ #	+8.6
Ammonia, "	+3.5	+0.3	—0.4

Experimental conditions correspond to those described in Table 2.

\* Prepared by the action of dilute enzyme solution (0.1 ml. per ml.) on 10  $\mu\text{M}$ . of urocanic acid.

<sup>†</sup> 10  $\mu\text{M}$ . of synthetic  $\alpha$ -L-formamidinoglutaric acid kindly supplied by Dr. Heinrich Waelsch.

# The presence of free glutamic acid (about 5  $\mu\text{moles}$  in experiment 1, and 8  $\mu\text{moles}$  in experiment 2) was demonstrated by paper chromatography.

<sup>3</sup> We are indebted to Dr. Heinrich Waelsch for sending us a sample of their compound.

These results suggest that in both *A. aerogenes* and *P. fluorescens* urocanic acid is converted to the same product, which is identical with the end-product of the degradation of histidine and urocanic acid in cat liver. The breakdown of this compound by *A. aerogenes* produces glutamic acid and formamide; and by *P. fluorescens*, glutamic acid, formic acid and ammonia (11). The experiments summarized in Table 3 support these assumptions. The intermediates produced by the dilute *Aerobacter* and *Pseudomonas* preparations were treated with concentrated preparations of *P. fluorescens* and of *A. aerogenes*, respectively. The end-products were identical with those produced by the action of the concentrated enzymes of the two strains on urocanic acid. Both preparations produced free glutamic acid, but only the *Pseudomonas* preparation produced free ammonia. Synthetic  $\alpha$ -L-formamidinoglutaric acid, kindly given us by Dr. Heinrich Waelsch, was similarly hydrolyzed by the *Aerobacter* extract to glutamic acid without the production of ammonia (Table 3, Exp. 3).

Neither *Aerobacter* extracts nor *Pseudomonas* extracts can hydrolyze formamide, and consequently this compound may well be the end-product of the hydrolysis of  $\alpha$ -L-formamidinoglutaric acid by *A. aerogenes*, but it cannot be an intermediate of its hydrolysis by *P. fluorescens*. Recent work by Tabor and Mehler (12) has provided evidence that extracts of this organism contain an enzyme which hydrolyzes  $\alpha$ -L-formamidinoglutaric acid to ammonia and N-formyl-L-glutamic acid, and that the latter compound is then split by another enzyme to formic acid and L-glutamic acid. A similar pathway was discovered in an unnamed soil organism by Suda and his collaborators (10). The experiment illustrated in Fig. 1 shows clearly that *Pseudomonas* extracts hydrolyze urocanic acid with the production of  $\alpha$ -L-formamidinoglutaric acid and of glutamic acid, and hydrolyze N-formyl-L-glutamic acid to glutamic acid. *Aerobacter* extracts, on the other hand, produce glutamic acid from urocanic acid, but not from N-formyl-L-glutamic acid. In this organism the enzymatic hydrolysis of  $\alpha$ -L-formamidinoglutaric acid leads therefore directly to glutamic acid and formamide, as shown in Fig. 2.



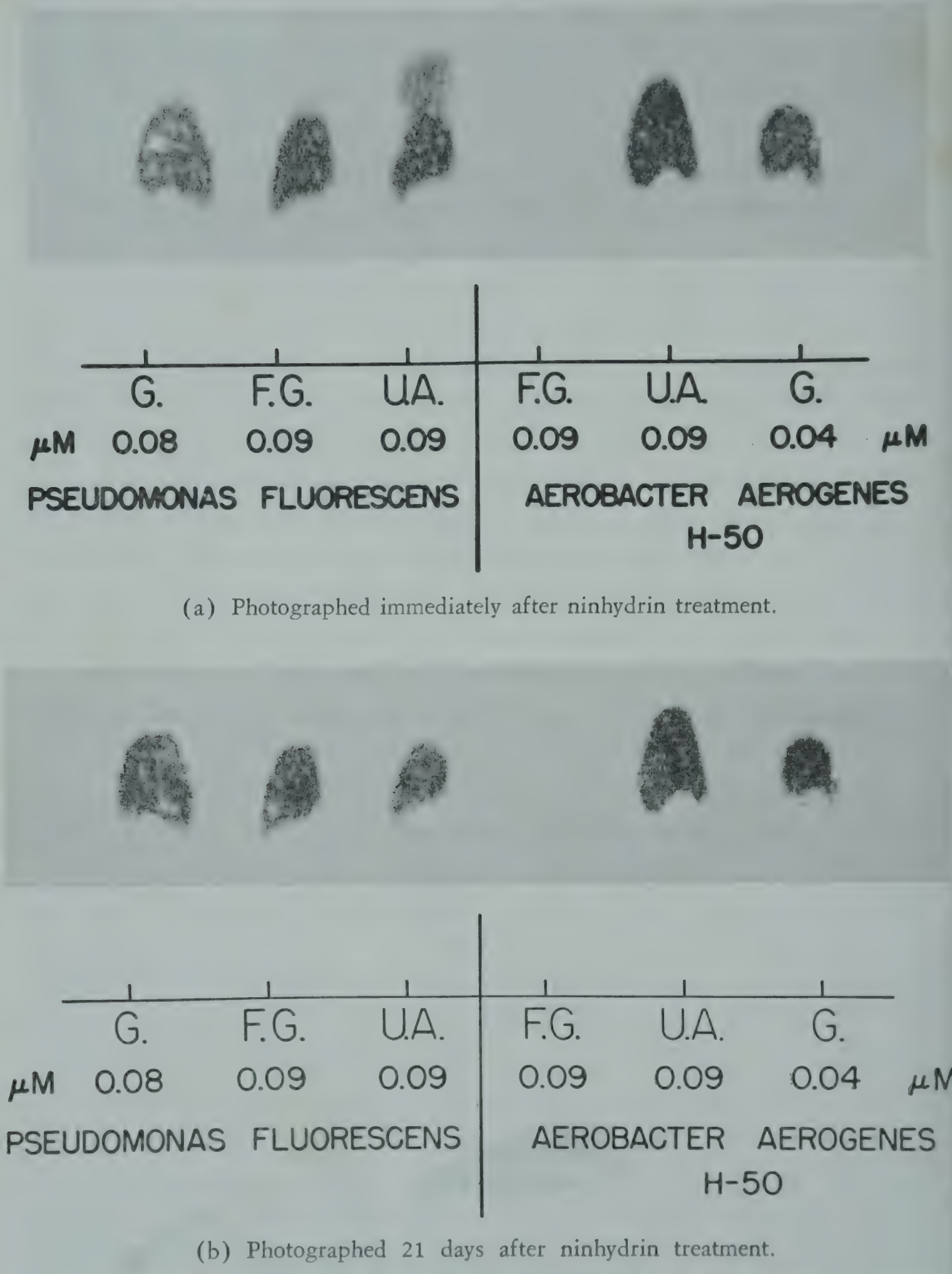
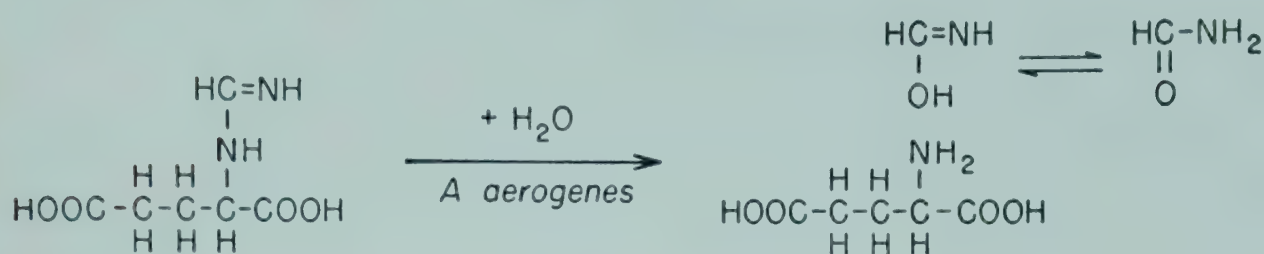


FIG. 1. Paper chromatogram, treated with ninhydrin, showing the action of *Pseudomonas* and of *Aerobacter* extracts on urocanic acid and on N-formyl-L-glutamic acid. G, glutamic acid; F. A., N-formyl-L-glutamic acid; U. A., urocanic acid. The experimental conditions correspond to those described in Table 2, Exp. 3 and 4. Solvent system: Butanol formic acid.

FIG. 2. Hydrolysis of  $\alpha$ -L-formamidinoglutaric acid by *A. aerogenes*.

## CONCLUSIONS

The observations reported here show that histidine-adapted *A. aerogenes* degrades histidine to glutamic acid and formamide. The glutamic acid is metabolized by resting cell preparations with the assimilation of a portion of the breakdown products, while the formamide is not further attacked. The pathways of histidine degradation of *A. aerogenes* and of *P. fluorescens* are illustrated in Fig. 3.

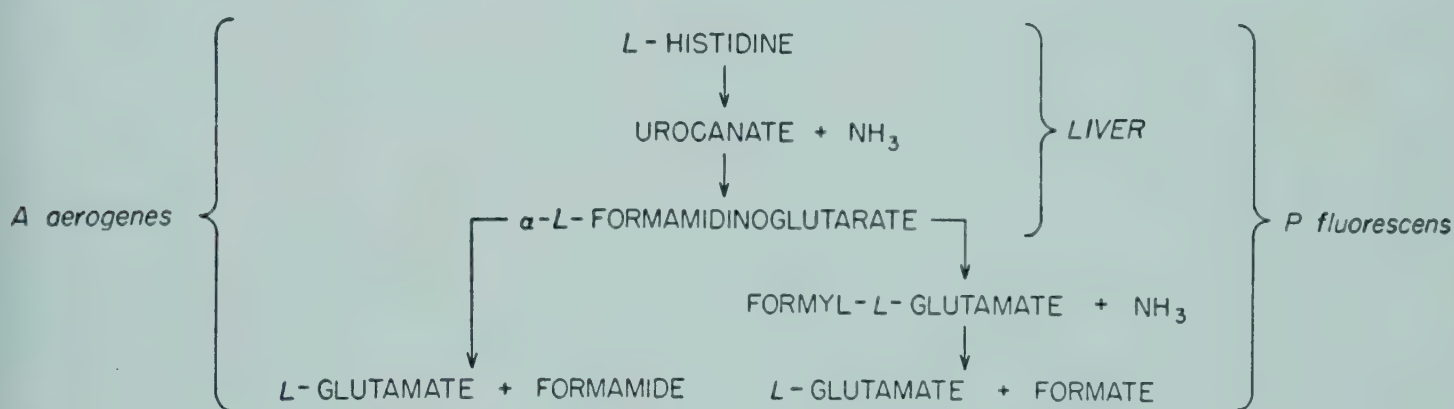


FIG. 3. Pathways of histidine degradation.

Both organisms, as well as liver preparations (2), produce  $\alpha$ -L-formamidinoglutaric acid via urocanic acid. This is converted to N-formyl-L-glutamic acid and ammonia by *P. fluorescens*, and to glutamic acid and formamide by *A. aerogenes*. The nature of the enzyme in *Aerobacter* extracts responsible for this reaction and the biological role of formamide are under investigation.

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# CERTAIN ASPECTS OF THE ENZYMATIC BREAKDOWN OF HISTIDINE \*

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OUR INTEREST in the enzymatic degradation of histidine was stimulated some years ago by reports that formylglutamines were intermediates on the metabolic pathway to glutamic acid, ammonia, and formic acid. Edlbacher (1) suggested that  $\delta$ -N-formylglutamine and Sera and his associates (2, 3) that  $\alpha$ -N-formylisoglutamine was the intermediate. The finding by Sera and Yada (4), and by Tabor and his group (5, 6) that urocanic acid was the first intermediate of enzymatic histidine breakdown made the formation of  $\delta$ -formylglutamine improbable but did not invalidate the possibility of formylisoglutamine being derived from histidine through urocanic acid. It appeared to us that the incomplete evidence presented for the occurrence of  $\alpha$ -N-formylisoglutamine, and in particular the lack of a comparison with a synthetic compound, made it desirable to reinvestigate the question. The intermediate which we isolated (7, 8) from cat liver extracts incubated with histidine or urocanic acid had properties which made us suggest that we were dealing with  $\alpha$ -formamidinoglutaric acid, a compound which had been postulated by Walker and Schmidt (9) solely on the basis of the appearance of an acidic group upon incubation of histidine with histidase extract. Not much can be added to our published reports (7, 8)

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on the isolation and properties of this compound and to what Tabor has reported. It should be stressed that the intermediate was isolated from the incubation mixture of histidine, as well as of urocanic acid, with cat liver extract. This was taken as additional proof that urocanic acid lies on the direct pathway of histidine breakdown. Our intermediate was isolated as the free acid by Dr. Blanche A. Borek, by one of the classical isolation procedures of precipitation with mercury acetate at pH 6.0 and decomposition of the mercury salt with hydrogen sulfide. Although we felt that the properties of the isolated compound were best explained by the assumption that we were dealing with the hydrate of  $\alpha$ -formamidinoglutaric acid there were two questions which had to be answered before the general significance of  $\alpha$ -formamidinoglutaric acid as intermediate in the enzymatic histidine breakdown could be accepted. First, the knowledge of the properties of the synthetic compound was needed for definite identification of the isolated natural product. Secondly, it had to be shown that we were not dealing with a pathway for histidine or urocanic acid metabolism occurring only in cat liver extracts.

Since the Bethesda group has now isolated the same compound from incubation mixtures of urocanic acid with extracts of guinea pig liver (10), it appears that one pathway of histidine metabolism in mammals leads to  $\alpha$ -formamidinoglutaric acid. We have found that a compound containing one equivalent of alkali-labile ammonia also accumulated when urocanic acid was incubated with extracts of histidine-adapted *Pseudomonas fluorescens*. In view of the rapid degradation of formamidinoglutaric acid by this extract, one may suspect that we are dealing here with the same compound.

The simultaneous synthesis by the Bethesda group (11) and in our laboratory (12) of  $\alpha$ -formamidinoglutaric acid with properties identical with that of the natural product resolved the first question. The properties of the natural and of the synthetic product are compared in Table 1. Of some interest appeared to be the pK's of the different functional groups of the compounds, since to our knowledge they are the first determinations carried out on formamidino groups.

TABLE 1  
 PROPERTIES OF  $\alpha$ -L-FORMAMIDINOGLUTARIC ACID  
 A, synthetic; B, from enzymatic digests.

Calc. for	Found	
$C_6H_{10}N_2O_4 \cdot H_2O$	A	B
C 37.5	37.2	37.5, 39.6
H 6.3	—	6.5, 6.7
N 14.6	14.6	14.0, 14.1
alkali-labile N		
7.3	7.3	7.0
$pK'_1; pK'_2; pK'_3$	2.7, 4.4, 11.3	2.4, 4.7, 11.1
$[\alpha]_D^{28}$	-10.3	-10.7
m. p.	85-95° C.	80-87° C.
infra-red spectrum	same	same

For comparative purposes we synthesized formamidinoacetic acid with a  $pK'_1$  2.6 and  $pK'_2$  11.5. It may be mentioned that all efforts up to the present to derivatize this group have failed; in particular, acylation attempts in weak alkali were unsuccessful, a situation reminiscent of the behavior of the guanidino groups. Both the natural and the synthetic product were degraded by extracts of histidine-adapted *Pseudomonas fluorescens* at a faster rate than histidine or urocanic acid.

Although this finding showed that formamidinoglutaric acid was an intermediate in histidine breakdown, the claim for formylisoglutamine deserved closer scrutiny. We therefore synthesized this compound (7, 8) and found that it was not decomposed by *Pseudomonas* extracts and that it had of course very different properties from formamidinoglutaric acid. It had been claimed that the formylisoglutamine isolated from enzymatic digests was optically inactive (3). The synthetic product was optically active with  $[\alpha]_D^{28} = -11.8$  in 2 N HCl (8).

On the basis of our isolation of formamidinoglutaric acid we formulated a sequence of events leading from histidine to this compound (Fig. 1) (8). The same sequence was suggested by Suda et al.



(13), who studied the breakdown of formylglutamic acid by histidine-adapted *Pseudomonas fluorescens*. They suggested formamidoglutaric acid as the intermediate preceding formylglutamic acid.

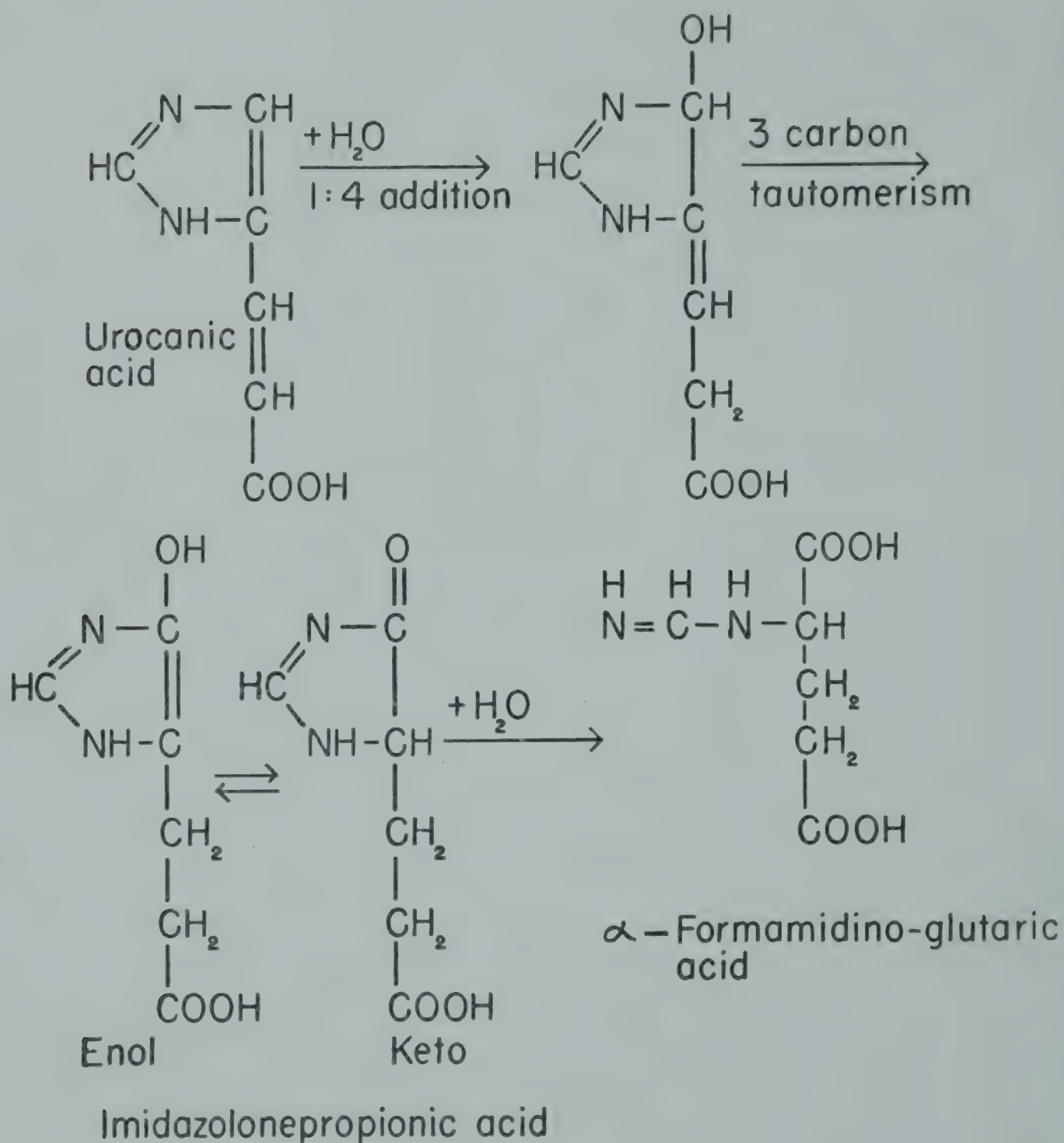


FIG. 1.

This scheme is in all essential points the same as that presented in this symposium by Tabor.

On the basis of known reactions of organic chemistry, we have suggested a mechanism for the conversion of urocanic acid to imidazolone propionic acid by way of a 1:4 addition of water followed by a 3-carbon tautomerism. We do not hold any particular

brief for the proposed mechanism, but if occurring it is probable that only the first step will be enzymatically catalyzed. The two steps lead to the enol form of imidazolone propionic acid, the compound which in its keto form is postulated to be the direct precursor of  $\alpha$ -formamidinoglutaric acid. The properties of this compound have a direct bearing on the identification of formamidinoglutaric acid, since it could be visualized that the phenolic group of the enol form would buffer in the alkaline region and that the imino group could simulate the properties of a weak acid. It is very probable that on the basis of the titration data, particularly that of formamidinoacetic acid, the different functional groups have been properly assigned in  $\alpha$ -formamidinoglutaric acid. As shown in Table 1, the synthetic  $\alpha$ -formamidinoglutaric acid is optically active and shows the same rotation as the natural product. L-Glutamic acid was isolated from the natural product upon hydrolysis (8). It is highly unlikely that imidazolone propionic acid would be optically active. Unfortunately we are dealing, as regards imidazolone propionic acid and formamidinoglutaric acid, with little-known groups of compounds. A perusal of the heroic compilation by K. Hoffman (14) of imidazole and its derivatives demonstrates that there are hardly any imidazolones known in which the carbon atom #2 is unsubstituted. We have made several attempts to synthesize imidazolone propionic acid, such as the desulfurization of the thiohydantoin of glutamic acid, ring closure of the benzyl ester of formamidinoglutaric acid, and ring closure of the free compound in acid, all of which up to the present were unsuccessful. Although compounds could be isolated which had the right carbon and nitrogen values, they were not decomposed by *Pseudomonas* extracts. It should be noted that in these reactions a number of products are formed and that we may have missed the right compound and concentrated on the one most easily isolated. A knowledge of the properties of the imidazolone propionic acid is of major interest not only because of its probable role as the precursor of formamidinoglutaric acid. This compound could also be the mother substance of a  $\alpha$ -N-formylisoglutamine, the isolation of which has been claimed by Sera and others from digests of guinea



pig liver and histidine. The question arises whether the formation of  $\alpha$ -N-formylisoglutamine from imidazolone propionic acid has to be considered an enzymatic process.

Kjaer (15) has found that upon boiling with water 2-benzylimidazolone is converted into phenylacetylglucinamide. In the preparation of the formylisoglutamine the Japanese authors (2, 3) deproteinized by boiling the solution, and the question arises whether we are not dealing here with a non-enzymatic cleavage of imidazolone propionic acid (Fig. 2). It may be mentioned that in our laboratory

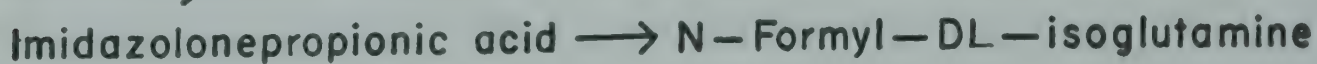
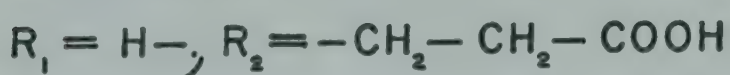
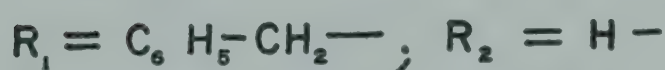
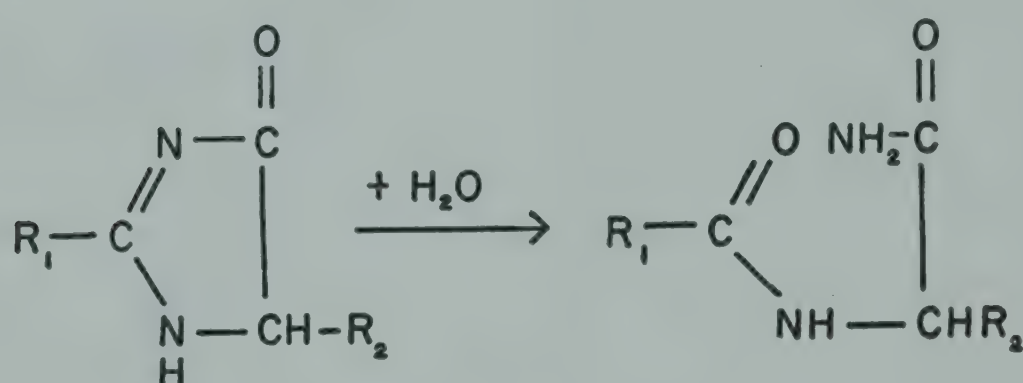


FIG. 2.

attempts to convert formamidinoglutaric acid into formylisoglutamine have been unsuccessful so far. In order to yield this product, formamidinoglutaric acid would have to cyclize to imidazolone propionic acid, a reaction which we have been unable to obtain up to now. In considering the metabolic pathway of histidine through formamidinoglutaric acid, as depicted in the scheme presented and supported by the available, well-documented evidence, one is impressed with one fact which has been uppermost in our minds in trying to understand the metabolic significance of this intermediate.

In our experiments in which histidine or urocanic acid was incubated with cat liver extracts a complete conversion of these compounds to "formamidinoglutaric acid" took place. It is more

accurate to state that histidine or urocanic acid was converted into a compound or compounds which contained one equivalent of alkali-labile ammonia. From the reaction mixture pure formamidinoglutamic acid was isolated in a yield corresponding roughly to 50 per cent of the alkali-labile ammonia. We do not know today whether the other 50 per cent was also formamidinoglutamic acid or possibly imidazolone propionic acid or some other compound with properties close to those of formamidinoglutamic acid. It is important to note that the enzymatic reaction stopped at compounds containing one equivalent of alkali-labile ammonia. Formamidinoglutamic acid itself was not further degraded by tissue slices or tissue homogenates of rat liver or guinea pig liver, although rapidly degraded by extracts of histidine-adapted *Pseudomonas fluorescens*. We, and Tabor's group (10) also, have interpreted this finding by stating that "the mammalian tissue preparation lacked the components necessary for further metabolism of the intermediate. These components may be either of catalytic nature or the acceptors for the one carbon unit of the amidine group" (8). In some preliminary experiments we have fortified homogenates with folic acid with and without a reducing milieu, and with leucovorin, but upon incubation with urocanic acid the reaction again failed to proceed beyond components containing one equivalent of alkali-labile ammonia, i. e. no free amino was liberated.

Although these findings are surely in no way evidence against formamidinoglutamic acid as an intermediate on the pathway of the metabolism of histidine as a donor of one-carbon units, they have made us speculate on possible other metabolic pathways of histidine. There are other data which may be recalled in this consideration. It was claimed recently that the enzyme, histidine deaminase, which converts histidine to urocanic acid is folic-acid-dependent. The enzyme preparation was obtained from guinea pig liver and after inactivation by long dialysis and adsorption could be reactivated by glutathione and folic acid (16). It is difficult to assess the validity of these experiments at the present time, but if reproducible they would introduce folic acid as a cofactor at an early stage of histidine



breakdown. Since we like to think of folic acid as being connected with the transfer of the one-carbon unit, one cannot help wondering whether the activation of carbon atom #2 of histidine does not occur as one of the first steps of its metabolism when it is acting as a donor of one-carbon units. The reactivity of the imidazole ring is indicated by the occurrence of ergothionine, 1- and 3-methyl-histidine, as well as spinacin (Fig. 3). This shows that all positions

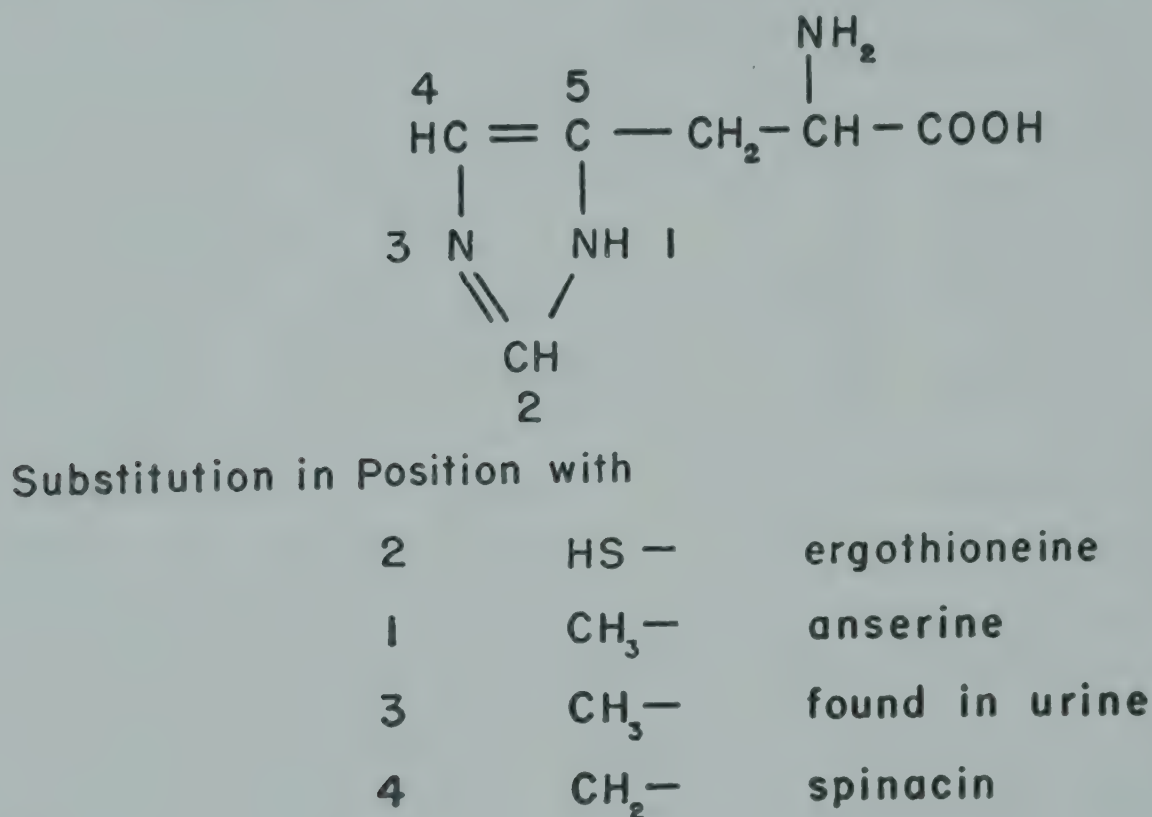


FIG. 3.

on the imidazole ring are subject to substitution and therefore can be activated enzymatically. One may speculate whether there are two pathways of histidine metabolism, one leading through urocanic acid and formamidinoglutaric acid to glutamic acid, formic acid, and ammonia, and another leading through an intermediate, still unknown, which acts as the donor of the one-carbon units. The activation of histidase by folic acid may suggest that this intermediate also may be the precursor of urocanic acid.

The adaptive nature of the enzyme system metabolizing histidine through formamidinoglutaric acid, as well as the excretion by folic-acid-deficient animals of degradation products of histidine (17) now also recognized as formamidinoglutaric acid (11), does not contradict such a mechanism.

To check the feasibility of this scheme, experiments with labeled urocanic acid and labeled formamidinoglutaric acid as donors of one-carbon units are being initiated.

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## DISCUSSION

DR. JAKOBY: I can't resist mentioning a case where *Neurospora* may be more like bacteria, rather than higher organisms. This I must admit is based on the barest minimum of evidence—namely, that there is in *Neurospora* an enzyme which can utilize formyl glutamate and seems to be specific for formyl glutamic acid—that is, formyl aspartic or formyl glycine are not attacked—which is less like liver and more like the *Pseudomonas* used in Dr. Tabor's experiments.

DR. COON: I would like to add a few comments to Dr. Ames' speculation on the origin of the 5-carbon chain of histidine. From our knowledge that histidine catabolism furnishes glutamate and formate and that formate is a precursor of carbon 2 of the amino acid in yeast, one might think that glutamic acid would also be a precursor. Dr. Levy found in our laboratory



that this is not the case and that glucose is a source of the carbon chain of histidine whereas acetate is not. Methyl-labeled acetate fed to yeast growing on a glucose medium yields radioactive glutamate, as one would anticipate, but the histidine formed is labeled only in carbon 2. Presumably, therefore, the methyl carbon of acetate furnishes formate at some stage in its metabolism. We have extended this work and found that glucose-6- $C^{14}$  and -1- $C^{14}$  yield labeled histidine and that the latter substrate furnishes histidine with about 50 per cent of the radioactivity of the carbon chain in carbon 5. This finding supports the suggestion that a triose-3- $C^{14}$  derived from the labeled glucose reacts with a  $C_2$  unit and the resulting pentose serves as precursor of the histidine carbon chain. The difficulty with this explanation is that the remaining 50 per cent of the  $C^{14}$  in the histidine chain is fairly equally distributed among the other carbons. The metabolic significance of this is not clear, particularly because our results with acetate rule out the possibility of partial randomization of the isotope via Krebs cycle intermediates prior to formation of the  $C_5$  chain.

I believe Dr. Ames made the comment that animals do not synthesize histidine, and I want to say that this statement should be modified if he includes man in this category. The nutritional evidence obtained by Dr. Rose and his students indicates that histidine is made by man, in contrast to all experimental animals so far studied. This was the stimulus for our initial study of this pathway in yeast. We have since found that labeled formate is incorporated into histidine in human liver slices and that this reaction does not occur in rat liver tissue. We have not eliminated the possibility of a formate-histidine exchange reaction in human liver, but it is certainly suggestive that this *in vitro* work parallels the nutritional data on man and the rat.

DR. WAELSCH: I would like to ask Dr. Tabor how well established the fact is of the non-reversibility of the histidine urocanic acid reaction. Has one ever done, for example, experiments to remove any formed histidine by a secondary reaction, for instance by using histidine carboxylase to pull the reaction over to histidine formation?

DR. TABOR: In spectrophotometric studies on the conversion of histidine to urocanic acid by purified histidase the reaction went to completion (within the limits of the spectrophotometric readings). Purified histidase preparations, furthermore, caused no disappearance of urocanic acid even in the presence of high concentrations of ammonium ions. Isotope experiments were also carried out in which labeled urocanic acid was incubated with histidase; in separate experiments unlabeled histidine carrier was added either before or after the incubation period. No isotope was found in the histidine fraction within the limits of our assay, but of course the reaction may be reversible to a smaller extent than this ( $<10^{-5}$ ).



A coupled reaction should certainly be attempted. The pH optimum of the bacterial decarboxylase, however, is about 4.5, while that for histidase is 8-9. Consequently this wouldn't be a good system for this purpose, but, perhaps better coupled systems will be found. This question of the reversibility of histidase is particularly interesting in view of its difference from the fumaric-aspartic system, where the equilibrium constant is roughly 1. One might speculate, of course, that the resonance of the urocanic acid molecule contributes more stability to this molecule, and this therefore might shift the equilibrium towards urocanic acid; however, as far as I know, no more definitive data on this aspect are available.

DR. ADELBERG: With regard to Dr. Coon's acetate experiments, Dr. Ehrensvaard reported in 1951 that the carboxyl group of histidine is derived exclusively from the methyl of acetate in experiments in which acetate is the sole carbon source. He found this in both yeast and *E. coli*. There appears to be some discrepancy there.

DR. COON: I don't think there is any discrepancy at all. Ehrensvaard's experiments were done with acetate as the sole carbon source, whereas our experiments involved the use of labeled acetate as a tracer with yeast grown on a glucose medium. In his experiments methyl-labeled acetate probably furnishes 1,2,5,6-labeled glucose prior to histidine formation.

DR. KNOX: There have been reports that the rates of the reactions degrading histidine are increased upon adaptation to histidine. We have been interested in increased rates of degradation of tryptophan in animal tissues upon adaptation to tryptophan, and I wonder if Dr. Tabor has done any experiments on adaptation to histidine in animals.

DR. TABOR: We have no definitive experiments on this point. However, there have been a number of experiments by Kapeller-Adler and others on other factors affecting histidase concentrations. Particular emphasis has been placed on the effects of various hormones in order to explain the histidinuria of pregnancy, but the results from the different laboratories are not in agreement. There have been some somewhat intriguing experiments published by Goryukhina, in which the liver activity was found to be increased in rabbits inoculated with a Brown-Pearce carcinoma, but not in animals with a methylcholanthrene tumor. In general, thus, little is known about the various factors, including adaptation, which may affect liver histidase in vivo.

DR. GRISOLIA: I would like to point out that some time ago when we found formyl glutamate was able to replace carbamyl glutamate for synthesis of Compound X, we had a chance through the kindness of Dr. Waelsch and Dr. Silverman to test their compounds in our system as possible precursors of Compound X. We obtained negative results. However, as I pointed out before in these meetings formyl, carbamyl, and related compounds can be



used for the synthesis of Compound X and for other routes of nitrogen metabolism. Although formamidino glutaric acid does not form Compound X as tested with the citrulline system, the possibility is open that it may prove to be related to formyl intermediates similar to the one active for citrulline synthesis but related to other synthetic routes.

DR. E. R. STADTMAN: Several months ago we were investigating the oxidation of several substrates by extracts of *Clostridium kluveri*. We observed that imidazole, urocanic acid, and histidine were very rapidly oxidized in the presence of versene and glutathione as catalysts. After a considerable bit of work, particularly on urocanic acid, which was a very good substrate, we identified the imidazole aldehyde as one of the major end products in this reaction. After additional work, we thought about carrying out a boiled enzyme control experiment and found that the reaction went equally well under these conditions. The interesting thing is that no oxidation occurred in the absence of versene or in the absence of glutathione or in the absence of boiled extract. This boiled extract could be substituted by a metal ion, either iron or cobalt. Of course, there are a few lessons to be gained from this series of experiments. One is that a boiled enzyme control should be included before so much work is done. The other is that the common practice of adding versene to enzyme reaction mixtures to protect against the oxidation of glutathione or other mercaptans is not a good procedure, since, in the presence of trace metals a very good artificial oxidizing system is thus obtained which may lead to nonenzymatic oxidations.

# THE BIOSYNTHESIS OF ISOLEUCINE, VALINE, AND LEUCINE<sup>1</sup>

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## THE BIOSYNTHESIS OF ISOLEUCINE AND VALINE

THE PROBLEM of the biosynthesis of the branched-chain amino acids has been under investigation in several laboratories for the past few years. These studies began in earnest with some experiments by Bonner, Tatum, and Beadle (8) using a *Neurospora* mutant requiring isoleucine and valine for growth. Bonner, in a later paper (7), suggested that the double requirement was due to the accumulation of an isoleucine precursor which then inhibited the conversion of an analogous intermediate in the path of valine synthesis. This interpretation, while ingenious and undoubtedly applicable in other pathways, has been shown to be incorrect in the case of isoleucine and valine, as discussed below.

Fig. 1 presents a summary of our concepts concerning the final steps in the biosynthesis of isoleucine and valine.

That the amino acids are formed by transamination of the corresponding keto acids (KI and KV) has been deduced from the following evidence: mutants of *Escherichia coli* are known which require isoleucine and valine for maximal growth, and which accumulate both keto acids (18). Neither the keto acids nor any other compounds can substitute for the growth requirements of these mutants, except as noted below. Other mutants, however, can use the keto acids in place of the amino acids (17). Wild-type *E. coli* contains a transaminase which catalyzes amino transfer between any two of

<sup>1</sup> Unpublished experiments of the author referred to in this paper were supported by a contract between the Regents of the University of California and the Office of Naval Research.



the following: isoleucine, valine, leucine, norleucine, norvaline, and glutamic acid (13). This enzyme is completely absent in the keto-acid-accumulating mutants (5, 13), and accounts for their inability to synthesize adequate amounts of isoleucine and valine. Both the wild-type and the mutants, however, contain an enzyme transaminating valine with either alanine or  $\alpha$ -aminobutyric acid; the presence

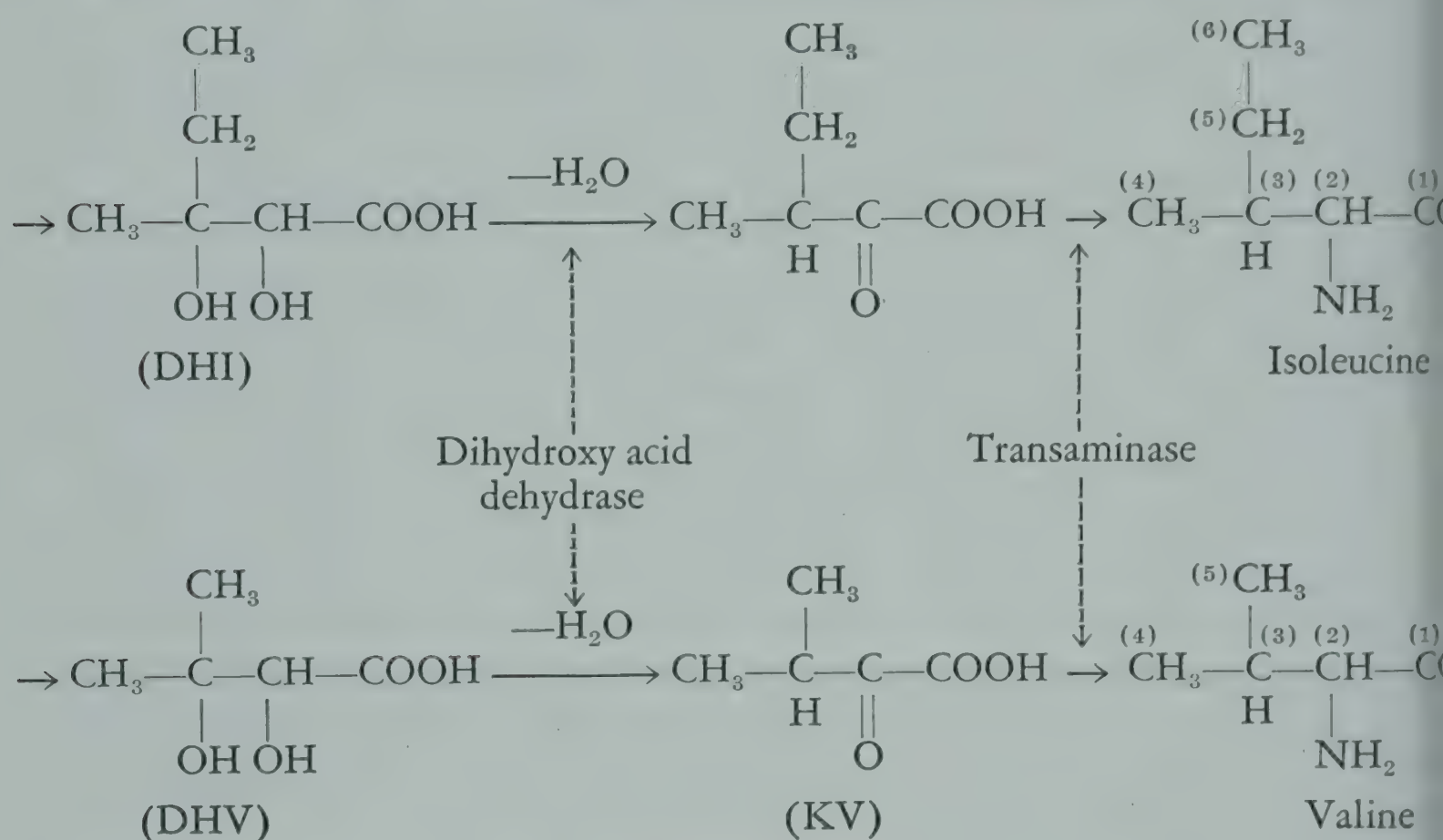


FIG. 1.

of this enzyme permits a limited synthesis of valine in the mutants, so that these mutants grow slowly without added valine, and rapidly with either valine, alanine, or  $\alpha$ -aminobutyric acid (5, 13). The latter two compounds act by increasing the rate of transamination of  $\alpha$ -ketoisovaleric acid to valine. From one such mutant, Adelberg and Umbarger (5) obtained a new strain capable of maximal growth on isoleucine alone, and discovered that the organism had mutated so as to increase its valine-alanine transaminase activity 4-5 fold.

The existence of the dihydroxy acid precursors (DHI and DHV) of isoleucine and valine was discovered by Adelberg and coworkers (3, 4). Evidence that they are normal precursors may be summarized as follows: they are accumulated by *Neurospora* and *E. coli* mutants

which require isoleucine and valine or their respective keto acids for growth (12); they can be used in place of the corresponding amino acids by mutants blocked earlier in the biosynthetic pathways (17); and the enzyme system dehydrating the dihydroxy acids to the keto acids has been extracted from wild type *E. coli* and *Neurospora* (12). Mutants accumulating the dihydroxy acids lack this enzyme system. It is not yet established whether one or two enzymes are concerned, but the fact that both activities are lost as the consequence of a single gene mutation suggests that one enzyme functions for both biosyntheses.

Thus in all cases of isoleucine-valine deficiency analyzed, the double requirement has been shown to result from the loss of two biosynthetic functions, and not from metabolic interactions as proposed by Bonner.

#### ORIGIN OF THE DIHYDROXY ACIDS

Taking up first the case of isoleucine biosynthesis, it has been recognized for several years that L-threonine and related four-carbon compounds ( $\alpha$ -ketobutyrate,  $\alpha$ -aminobutyrate,  $\alpha$ -keto- $\beta$ -hydroxybutyrate, D-threonine) can replace isoleucine in the nutrition of many mutant microorganisms (15, 16, 17). While it is clear that these compounds are either normal biosynthetic intermediates or are convertible to such, their exact positions in the scheme have not been conclusively established. The concept that L-threonine itself is an isoleucine precursor has been strengthened (though by no means proved) by several types of experimental evidence:

(a) A double mutant of *Neurospora* has been prepared, combining a strain unable to synthesize threonine and a strain accumulating DHI. Washed resting mycelial pads of the double mutant have been shown to convert added L-threonine to DHI in 50 per cent yield. When the experiment was carried out using  $C^{14}$ -1,2-threonine, the DHI formed was labeled in positions 1 and 2,\* with 73 per cent of the specific activity of the threonine (2).

\* The numbering system for isoleucine and valine carbons is shown in Fig. 1.



(b) In isotopic competition experiments with *E. coli* carried out by Abelson (1), threonine suppressed the radioactivity in isoleucine to one-third of the value of the control.

In 1951, Tatum and Adelberg (14) proposed a common biosynthetic origin for DHI and DHV, because of the striking similarity of the  $C^{14}$ -distribution in these two compounds when *Neurospora* was fed  $C^{14}$ -acetate. However, a common origin from threonine has now been ruled out, since in the experiments described above, added threonine had no effect either on the DHV excreted by the *Neurospora* double mutant or on the radioactivity of valine in the isotopic competition experiments.

The carbon skeleton of valine thus does not arise from threonine or from four-carbon compounds derived from threonine. That a part of the carbon skeletons of isoleucine and valine may have a common origin from some other intermediate, however, will be shown later in this discussion.

Coming back to the origin of DHI, the experiments mentioned earlier establish that threonine can furnish the carboxyl and alpha carbons of isoleucine. However, the conversion of the four-carbon chain of threonine to the six-carbon branched chain of isoleucine has been something of an enigma, since Ehrensvar (10, 11) has found these two compounds to be derived from acetate in the aerobic metabolism of several microorganisms, as shown in Fig. 2. Fig. 2 shows that there is no four-carbon sequence in the isoleucine skeleton corresponding to that of threonine. Two possibilities are (a) that threonine furnishes carbons 1-3, or (b) that threonine furnishes two  $C_2$  fragments which eventually appear as carbon 1-2 and 5-6 of isoleucine. The latter possibility appeared attractive, since carbon 4 of threonine and carbon 6 of isoleucine are the only known cases where an amino acid methyl group is derived from acetate carboxyl; and whereas the enzymatic mechanism for this reduction is known in the case of threonine (6), there is no similar information on a reduction for an isoleucine carbon.

To help solve the enigma, I have carried out isotopic competition experiments using the *Neurospora* mutant that accumulates the

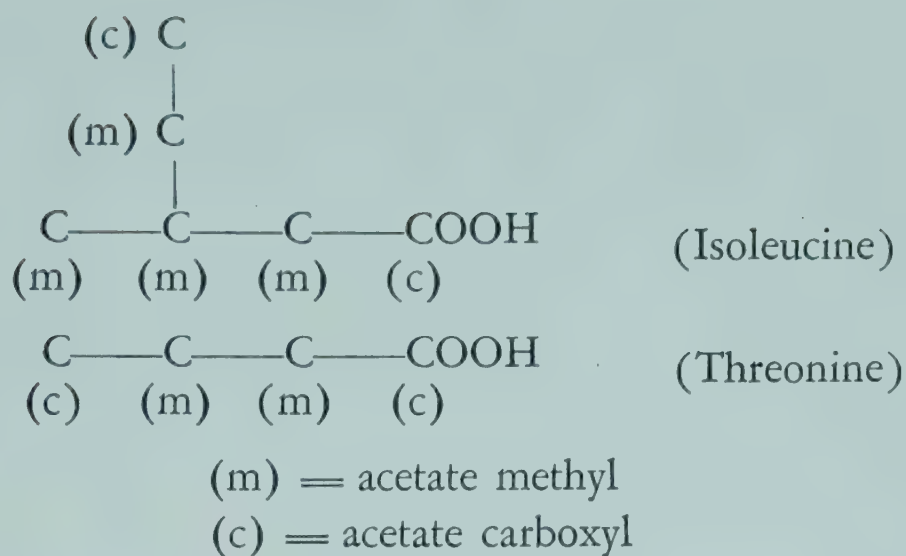
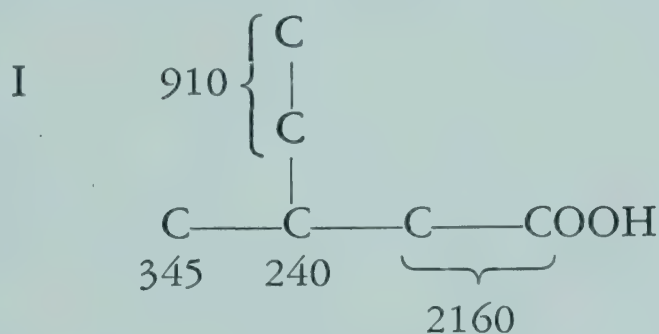
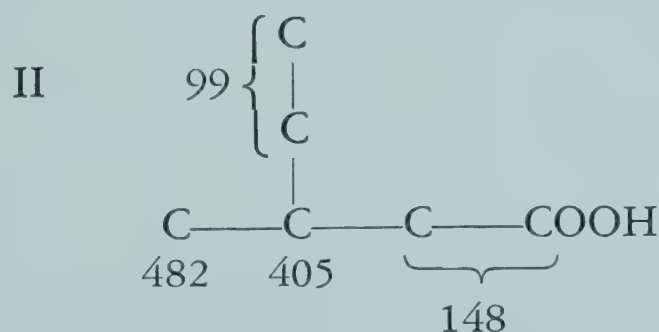


FIG. 2.

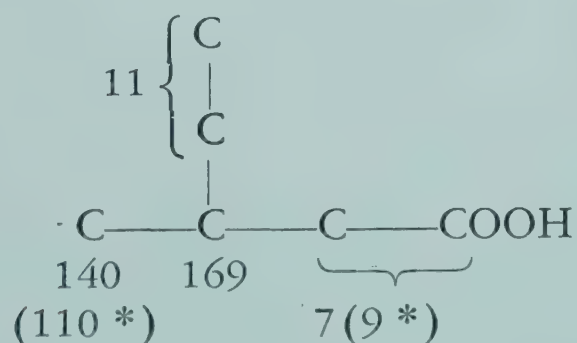
DHI formed from  $\text{C}^{14}$ -2-Acetate; cpm/ $\mu\text{M}$ .:



Same conditions as in I, but unlabeled L-Threonine present:



Effect of threonine on  $\text{C}^{14}$  incorporation;  $[\text{II}/\text{I}] \cdot 100$ :



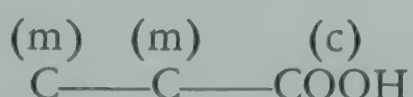
\* Rechecked in second experiment with  $\text{C}^{14}$ -1,2-Ac.

FIG. 3.



dihydroxy acids (2). One resting mycelium of this mutant was fed  $C^{14}$ -2-acetate, and a second mycelium was fed the same quantity of labeled acetate together with 100  $\mu M$ . of non-labeled L-threonine. The dihydroxy acids produced were isolated and degraded, with the results shown in Fig. 3. The results show that threonine furnishes carbons 1, 2, 5, and 6 of isoleucine. Since  $C^{14}$ -1,2-threonine was found, in the experiment mentioned earlier, to contribute labeling only to positions 1 and 2 of isoleucine, the contribution to positions 5 and 6 must come from threonine carbons 3 and 4.

The metabolic utilization of threonine in this manner could come about either (a) by a splitting of a four-carbon compound derived from threonine into two  $C_2$  fragments which are then added to the same carbon of a third compound; or (b) by some rearrangement mechanism such as takes place during the catabolism of phenylalanine and tyrosine. The four-carbon compound involved cannot be threonine itself, since it is well established that threonine is first deaminated to  $\alpha$ -ketobutyric acid before being used for isoleucine synthesis. It should also be noted (see Fig. 2) that the two carbons of isoleucine (positions 3 and 4) not furnished by threonine are derivable from two acetate methyl groups in Ehrensvar'd's experiments.  $C_2$  fragments of this derivation can come from pyruvic acid, since Ehrensvar'd (11) has shown that compounds formed from pyruvic acid (such as alanine) have the derivation:



These facts have been used to formulate a possible mechanism for the biosynthesis of DHI (Fig. 4). The first step in the hypothetical sequence would be an aldol condensation between  $\alpha$ -ketobutyric acid and pyruvic acid to form a  $C_7$  keto acid intermediate (I). Enolization and hydration of this compound would form a pinacol (II). Pinacols undergo rearrangements involving exchange of one hydroxyl with the alkyl group on the adjacent glycol carbon; while this has only been known to take place in the presence of electrophilic reagents (e. g., mineral acids) it is conceivable that it might proceed

enzymatically under physiological conditions. The pinacol rearrangement shown in Fig. 4 would result in a  $\beta$ -keto acid, which would then spontaneously decarboxylate to give compound III.

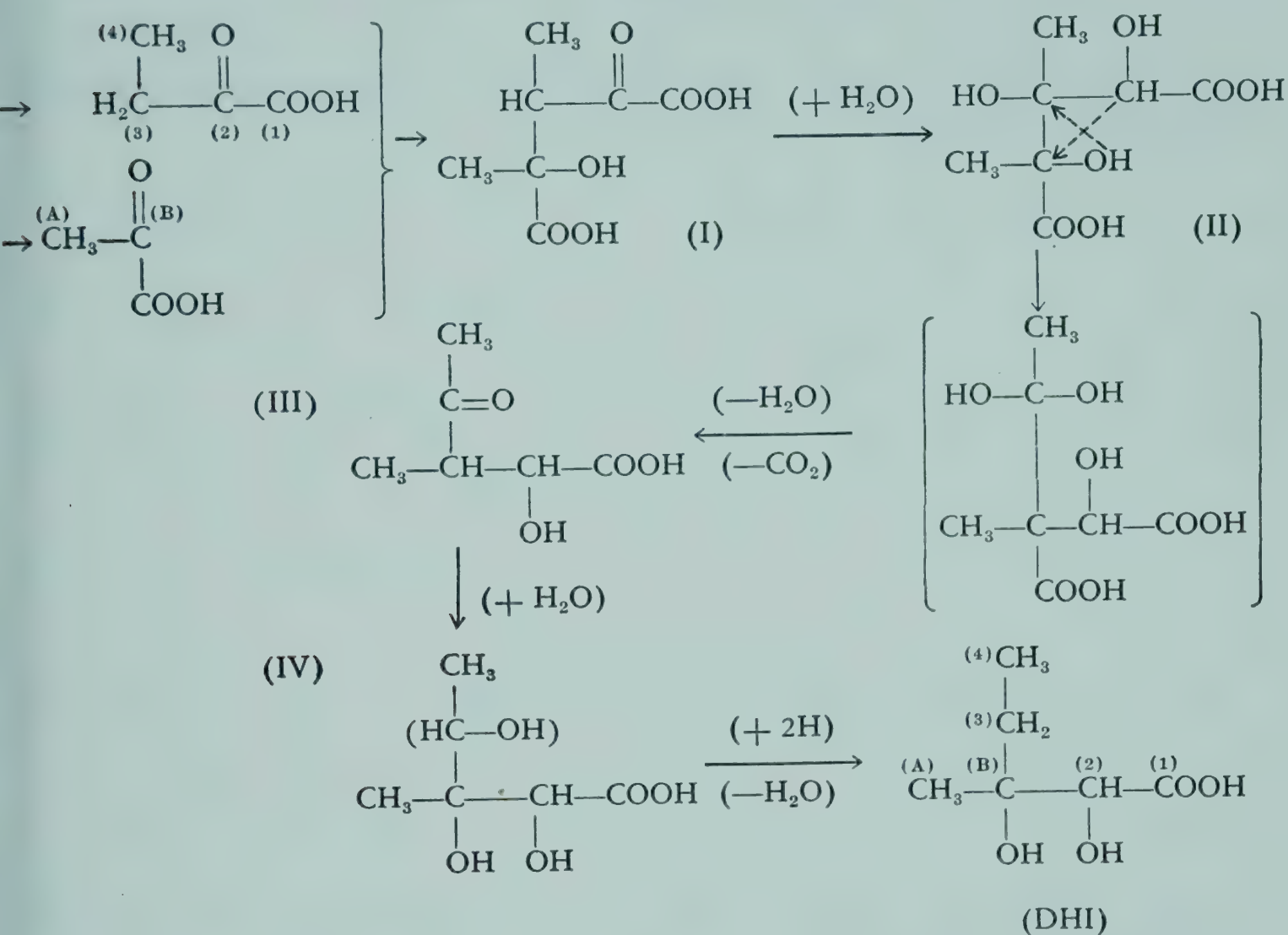


FIG. 4.

Enolization and hydration of III would form a tri-hydroxy acid (IV) with the carbon skeleton of isoleucine and a derivation compatible with all experimental data available. Further evidence for the formation of compound IV is the observation (2) that a mutant strain of *Neurospora*, requiring isoleucine and valine for growth, accumulates two lactones which are also glycols, one of which may conceivably have the structure proposed for compound IV.

It is possible that the decarboxylation of pyruvate precedes instead of follows the condensation, and it is also possible that a ketol condensation rather than an aldol condensation takes place prior to the



rearrangement. Much work remains to be done before we know which, if any, of these hypotheses is correct.

The biosynthesis of DHV poses a similar enigma. It has been shown (1, 9, 13a) that it probably is formed from pyruvic acid; however, Ehrensvar (10) has found that under anaerobic conditions the derivations of valine and of pyruvic acid in certain microorganisms are as shown in Fig. 5.

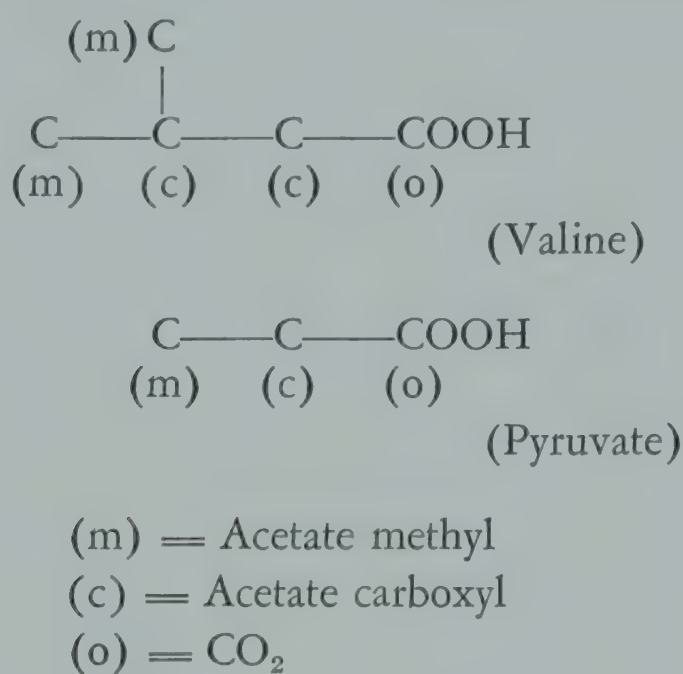


FIG. 5.

Once again, the sequence found in the precursor (in this case pyruvate) does not appear as such in the end-product. However, if the sequence of reactions shown in Fig. 4 were to proceed, with pyruvic acid substituted for  $\alpha$ -ketobutyric acid, the final product would be DHV with a carbon skeleton obtained totally from pyruvic acid and with derivations compatible with Ehrensvar's data (Fig. 5). Still another possibility is the reaction sequence presented in Fig. 6, which closely resembles the scheme proposed by Strassman, Thomas, and Weinhouse (13a).

According to the hypothetical sequences shown in Figs. 4 and 6, DHI and DHV are synthesized by a series of analogous reactions. This conclusion is also suggested by the fact that mutants of *Neurospora* are known which are blocked prior to the dihydroxy acid stage and which yet show the nutritional requirement for both isoleucine

and valine. It is tempting to speculate that the two lactone-glycols accumulated by one of these strains may have the trihydroxy-acid structures (IV and VII) shown in Figs. 4 and 6.

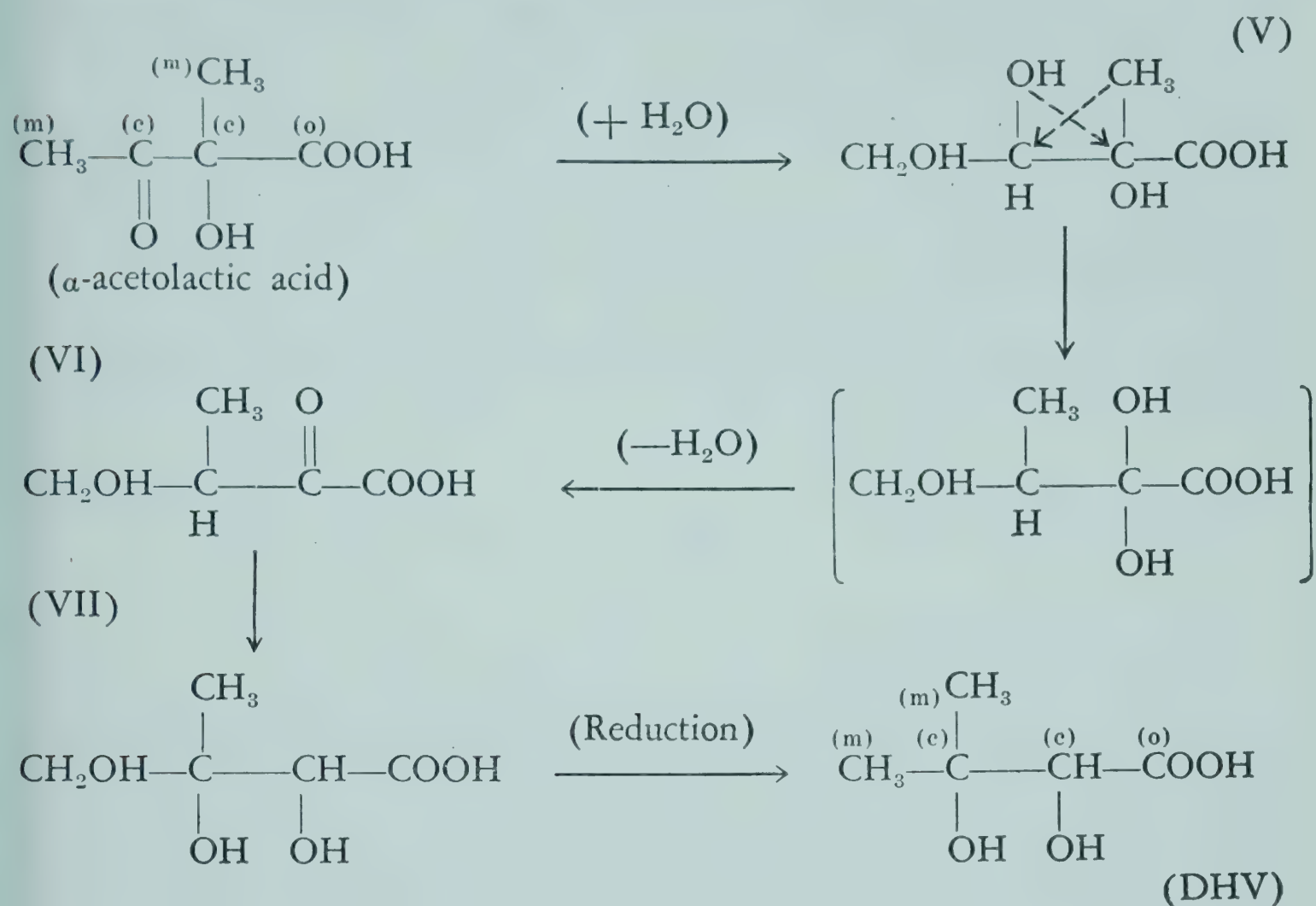


FIG. 6.

### BIOSYNTHESIS OF LEUCINE

Most of our knowledge concerning the origin of leucine comes from Abelson's isotopic competition experiments (1). His data suggest that the keto-acid corresponding to leucine (KL) is a precursor of leucine, and that KL is in turn formed from KV. This would involve loss of the KV carboxyl group and addition of a fragment derived from acetate to form carbons 1 and 2 of KL (Fig. 7).

This mechanism is compatible with Ehrensvar'd's data (10) concerning the derivations of valine and leucine (Fig. 8) and with his observation that leucine is exceptional in having a carboxyl derived directly from acetate rather than from respiratory cycle intermediates



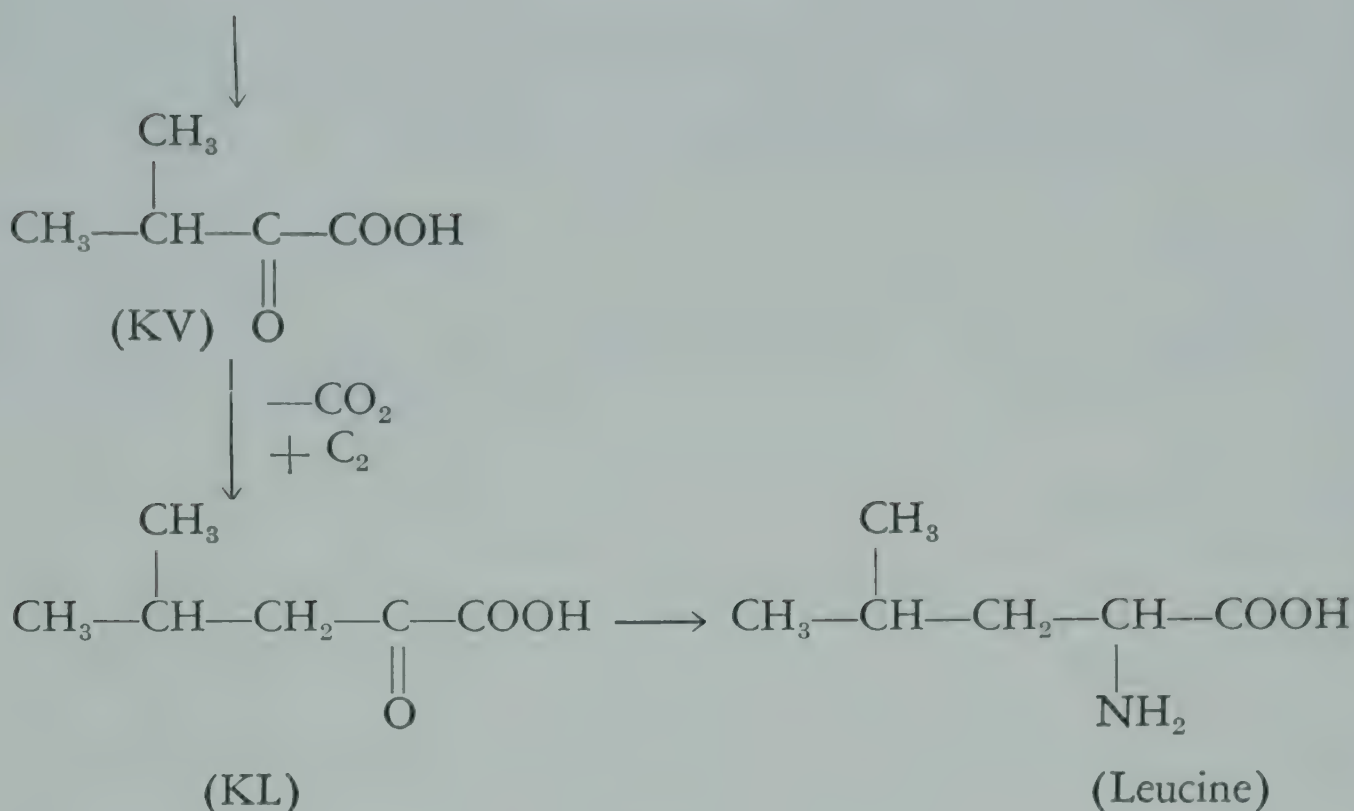
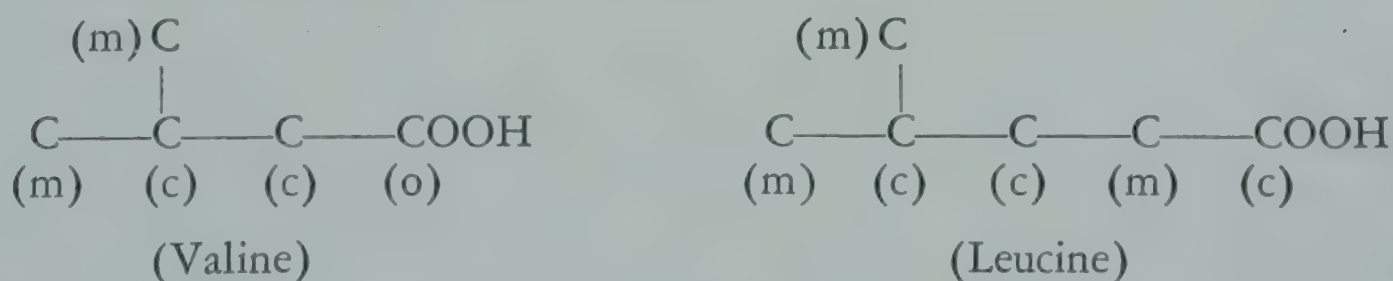


FIG. 7.

(11). No information has been published on the reactions involved in the conversion of KV to KL. The last step in leucine biosynthesis is probably a transamination of KL, since KL is known to be an active substrate for transaminations (13). If so, there must be more than one leucine transaminase (at least in *E. coli*), since mutational loss of the transaminase studied by Rudman and Meister (13) did not result in a nutritional requirement for leucine.

Anaerobic Derivations (Ehrensvar):



(m) = Acetate methyl  
 (c) = Acetate carboxyl  
 (o) = CO<sub>2</sub>

FIG. 8.

## SUMMARY

The following conclusions concerning the biosynthesis of isoleucine, valine, and leucine seem fairly well established:

(1) Valine and isoleucine are formed by transformations of the corresponding dihydroxy acids to the keto acids, followed by transamination to the amino acids.

(2) The dihydroxy acid precursor of isoleucine can obtain four of its carbons from L-threonine. Threonine carbons 1 and 2 become isoleucine carbons 1 and 2, but threonine carbons 3 and 4 become separated from 1 and 2 and appear in positions 5 and 6 of isoleucine (see Fig. 1 for numbering system).

(3) The dihydroxy acid precursor of valine is formed from pyruvic acid.

(4) Leucine is formed from the corresponding keto acid, which in turn is derived from the keto acid precursor of valine by decarboxylation and addition of an acetate-derived C<sub>2</sub> fragment.

Some speculation is presented concerning the mechanism of formation of the dihydroxy acid precursors of isoleucine and valine. In both cases, the isotopic data are compatible with a condensation reaction followed by a pinacol rearrangement.

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# ENZYMATIC STUDIES ON THE BIOLOGICAL DEGRADATION OF THE BRANCHED CHAIN AMINO ACIDS \*

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THE EVIDENCE to be presented fails to confirm the older view that the branched-chain amino acids undergo either reductive or oxidative demethylation at any stage of their metabolism. It does, however, support the idea that the  $\alpha$ -keto analogs of these compounds decarboxylate oxidatively to furnish branched-chain fatty acids (as Co-enzyme A thiol esters). The decarboxylation is presumably irreversible, as is the conversion of pyruvate to acetyl-CoA (23), thereby accounting for the inability of animals, including man, to accomplish the biosynthesis of these three amino acids from their degradation products (32).

## LEUCINE METABOLISM

Isovaleric acid has long been recognized as a probable intermediate in the degradation of leucine, the most strongly ketogenic of the amino acids (17, 29, 16, 10). More recent studies with isotopes have provided information on the mechanism by which these two compounds yield ketone bodies in animal tissues (6, 14, 45, 11) and have demonstrated that these reactions lead to the incorporation of carbon dioxide into the carboxyl group of acetoacetate (11). This discovery was extended by Plaut and Lardy (27), who provided evidence that the carbon dioxide fixation is stoichiometric in nature.

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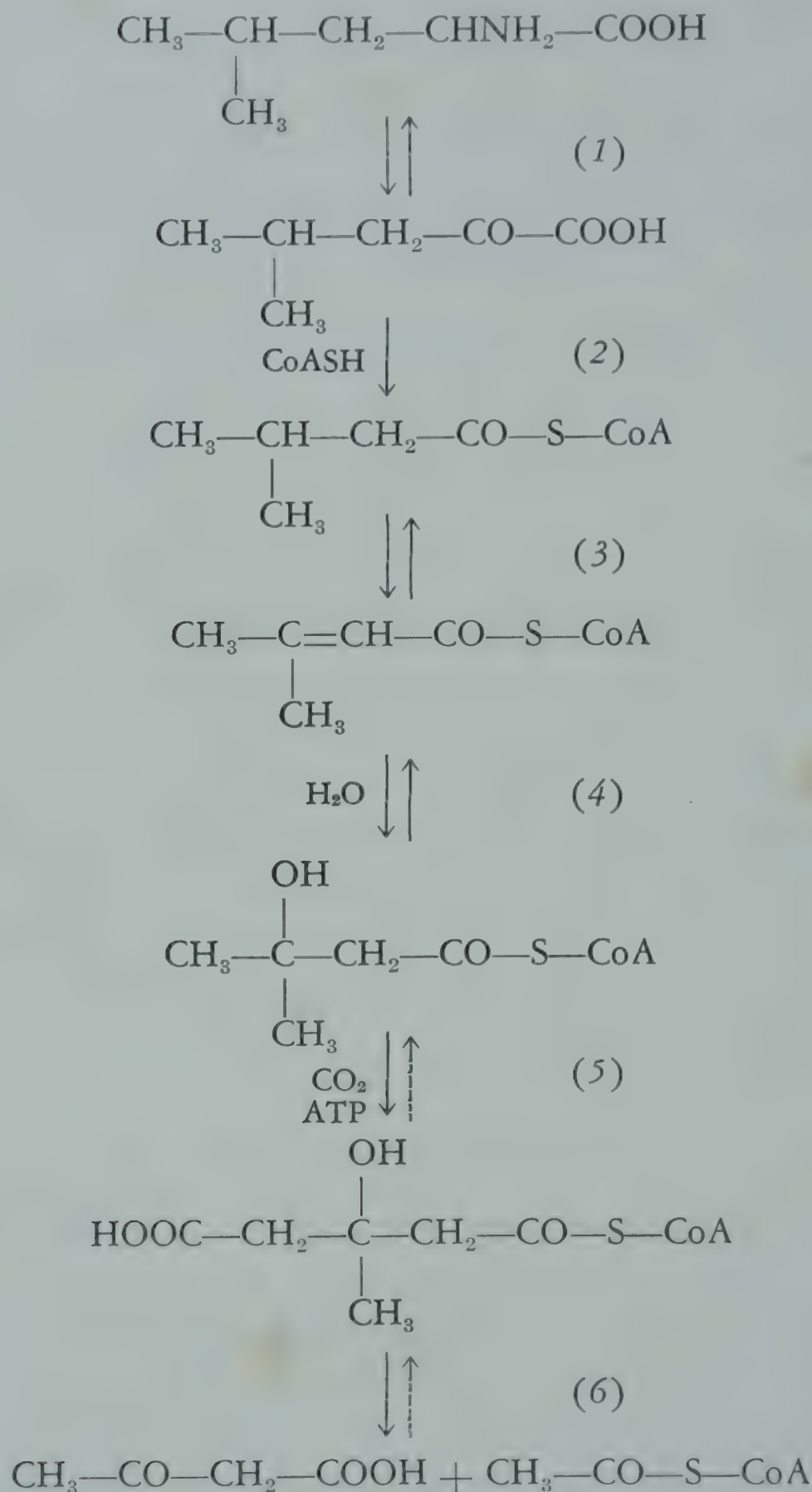


FIG. 1. Proposed scheme of leucine metabolism.

Recent work on this metabolic pathway in our laboratory has been concerned primarily with two enzymatic steps which are of particular interest because they are different from those of straight-chain fatty acid metabolism: carbon dioxide fixation, and cleavage of the carbon

chain without prior formation of a  $\beta$ -keto acid. The accompanying series of reactions is proposed to account for the results obtained (Fig. 1).

The oxidation of isovaleryl-CoA to senecioyl-CoA (Reaction 3) is postulated by analogy to the corresponding reaction with straight-chain fatty acids (35, 21). The finding that senecioic acid incorporates radioactive carbon dioxide into the carboxyl carbon of acetoacetate when incubated with ATP, CoA, and dialyzed extracts of rat or pigeon liver acetone powder suggests that the activation of the acid to form the CoA ester precedes further reactions (30). Direct evidence for the involvement of thiol esters has been provided by the demonstration that dialyzed heart extracts catalyze the conversion of senecioyl-CoA to  $\beta$ -hydroxyisovaleryl-CoA (Reaction 4) and fix labeled carbon dioxide into acetoacetate in the presence of either of these synthetically prepared substrates, whereas the corresponding free acids are inactive. The hydration step is apparently due to the presence of crotonase (39, 5) in these heart fractions, for highly purified liver crotonase<sup>1</sup> rapidly catalyzes this reaction. In order to determine which of these thiol esters is the primary substrate for the carboxylation step, it was necessary to free the heart system of crotonase. This was accomplished by brief heating at 60° C. at pH 7.4, and with the aid of this enzyme preparation  $\beta$ -hydroxyisovaleryl-CoA was established as the substrate for acetoacetate synthesis (2). As may be seen in Table 1, senecioyl-CoA is converted to acetoacetate to an appreciable extent only in the presence of added crotonase, whereas  $\beta$ -hydroxyisovaleryl-CoA does not require this supplement. The system requires the addition of ATP as well as bicarbonate.

The intermediate product predicted as a result of carbon dioxide fixation (Reaction 5) is  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA (HMG-CoA). The corresponding free acid was prepared by the method of Klosterman and Smith (22) and converted to the thiol ester by the

<sup>1</sup> Kindly furnished by Dr. Joseph R. Stern.



TABLE 1

ENZYMATIC SYNTHESIS OF ACETOACETATE FROM  $\beta$ -HYDROXYISOVALERYL-CoA

Substrate	System	Acetoacetate formed (Per cent of yield from $\beta$ -hydroxyiso- valeryl CoA in the complete system)
Senecioyl-CoA	Complete	8
Senecioyl-CoA	Complete + crotonase	80
$\beta$ -Hydroxyisovaleryl-CoA	Complete	100
$\beta$ -Hydroxyisovaleryl-CoA	KHCO <sub>3</sub> omitted	20
$\beta$ -Hydroxyisovaleryl-CoA	ATP omitted	0

The test system contained 500  $\mu$ M. Tris buffer, pH 8.1, 20  $\mu$ M. MgCl<sub>2</sub>, 26  $\mu$ M. cysteine, 200  $\mu$ M. KHCO<sub>3</sub>, 10  $\mu$ M. ATP, 1  $\mu$ M. CoA ester, pig heart enzyme fraction free of crotonase, and, where indicated, 0.01 mg. crystalline liver crotonase<sup>1</sup>; volume 4.2 ml., incubation 60 minutes at 38° C. Maximum yield represents 0.26  $\mu$ M. of acetoacetate, estimated by a modification of the method of Barkulis and Lehninger (4).

general method of Wieland and Rueff (43). This compound (but not the free acid) furnishes acetoacetate upon incubation with the enzyme system (Table 2). The cleavage (Reaction 6) requires the presence of cysteine (or glutathione), but, unlike the carboxylation reaction, is not dependent upon the addition of ATP and bicarbonate.

TABLE 2

ENZYMATIC CLEAVAGE OF  $\beta$ -HYDROXY- $\beta$ -METHYLGLUTARYL-CoA

Additions	Acetoacetate formed (Per cent of yield with cysteine added)
26 $\mu$ M. glutathione	113
26 $\mu$ M. cysteine	100
None	9
26 $\mu$ M. cysteine + 20 $\mu$ M. ATP + 500 $\mu$ M. KHCO <sub>3</sub>	87

The test system contained 200  $\mu$ M. Tris buffer, pH 8.1, 20  $\mu$ M. MgCl<sub>2</sub>, 0.5  $\mu$ M. HMG-CoA, and a fraction of pig heart precipitated by 32-50 per cent ethanol (0.49 mg. protein); volume 1.3 ml., incubation 60 minutes at 38° C.

The other product of the cleavage reaction has been identified chromatographically as acetyl-CoA. After incubation of HMG-CoA with the enzyme system described, the thiol esters present were converted to the hydroxamic acids (36) and submitted to paper chromatography in water-saturated phenol. The reaction product ( $R_f$  0.58) was identified by comparison with known acetohydroxamic acid (0.55) and HMG-hydroxamic acid (0.17). Additional proof for the products of Reaction 6 was obtained in an experiment in which HMG-CoA was incubated with the enzyme system and, after deproteinization, aliquots were taken for the independent determination of acetoacetate and acetyl-CoA (by citrate formation in the presence of oxalacetate and crystalline condensing enzyme<sup>1</sup> ref. 40). The molar ratio of acetyl-CoA to acetoacetate was 0.75 (2). The ratio is about 1.0 if a correction is made for the lability of acetyl-CoA under the conditions employed (incubation for 1 hour at 38° C., pH 8.1) prior to the deproteinization step. As anticipated, acetyl-CoA was found to yield no free acetoacetate under these conditions, for heart extracts require the presence of succinate to accomplish this conversion (38, 20).

The reaction scheme accounts for the strongly ketogenic properties of leucine, for the complete catabolism of the amino acid would lead to the formation of 1.5 moles of acetoacetate, assuming conversion of the acetyl-CoA to acetoacetate. It is particularly interesting that other investigators have shown the conversion of labeled acetate to  $\beta$ -hydroxy- $\beta$ -methylglutarate in liver preparations (28, 34) and are considering this reaction as a step in cholesterol synthesis (9). This might be considered as representing the reversal of Reaction 6, a process bearing a close analogy to the formation of citrate from acetyl-CoA and oxalacetate (40). The requirement for cholesterol synthesis of one or more of the branched chain acyl-CoA compounds shown in the reaction sequence would represent the only known metabolic role of leucine other than participation in protein synthesis.



## ISOLEUCINE METABOLISM

In sharp contrast to leucine, isoleucine and  $\alpha$ -methylbutyrate are only weakly ketogenic (3, 44, 7, 16, 24, 42) and have slight, but definite glycogenic properties (15, 7, 41). An investigation of the fate of  $\alpha$ -methylbutyrate- $\beta$ -C<sup>14</sup> in liver slices indicated the occurrence of  $\beta$ -oxidation on the longer carbon chain and cleavage to produce "acetate" from carbons 3 and 4 (12). This 2-carbon unit was found to give rise to acetoacetate. With a preparation of  $\alpha$ -methyl-C<sup>14</sup>-butyrate it was further shown that "propionate" is formed from the remaining carbons of the substrate (13).

The accompanying series of reactions (Fig. 2) is based on the scheme originally proposed (13) and on recent evidence that CoA esters are intermediates. A preparation of tiglic acid (the *cis* isomer of 2-methyl-2-butenic acid) was purified by sublimation and converted to the CoA thiol ester. The latter compound was found to undergo hydration (Reaction 10) in dialyzed alcohol-potassium-chloride extracts of pig heart or rat liver. When neutral hydroxylamine was added to trap the thiol esters in such a reaction mixture and the products were submitted to chromatography in water-saturated butanol, a new spot was obtained ( $R_f$  0.44) in addition to that of tiglohydroxamic acid ( $R_f$  0.84). The hydration is also catalyzed by crystalline liver crotonase (37). Evidence for the formation of acetyl-CoA from tiglyl CoA was obtained by measuring citrate formation in heart or liver extracts supplemented with DPN and oxalacetate (Table 3). A dialyzed ammonium sulfate fraction of the liver extract was almost completely dependent upon the presence of DPN. The postulated DPN-dependent oxidation (Reaction 11) and cleavage to furnish acetyl-CoA (Reaction 12) are analogous to enzymatic steps already recognized in straight-chain fatty acid metabolism (25).

The other product of Reaction 12 was identified in an experiment in which tiglyl-CoA was incubated with DPN, oxaloacetate, and the dialyzed ammonium sulfate fraction of rat liver extract, and the

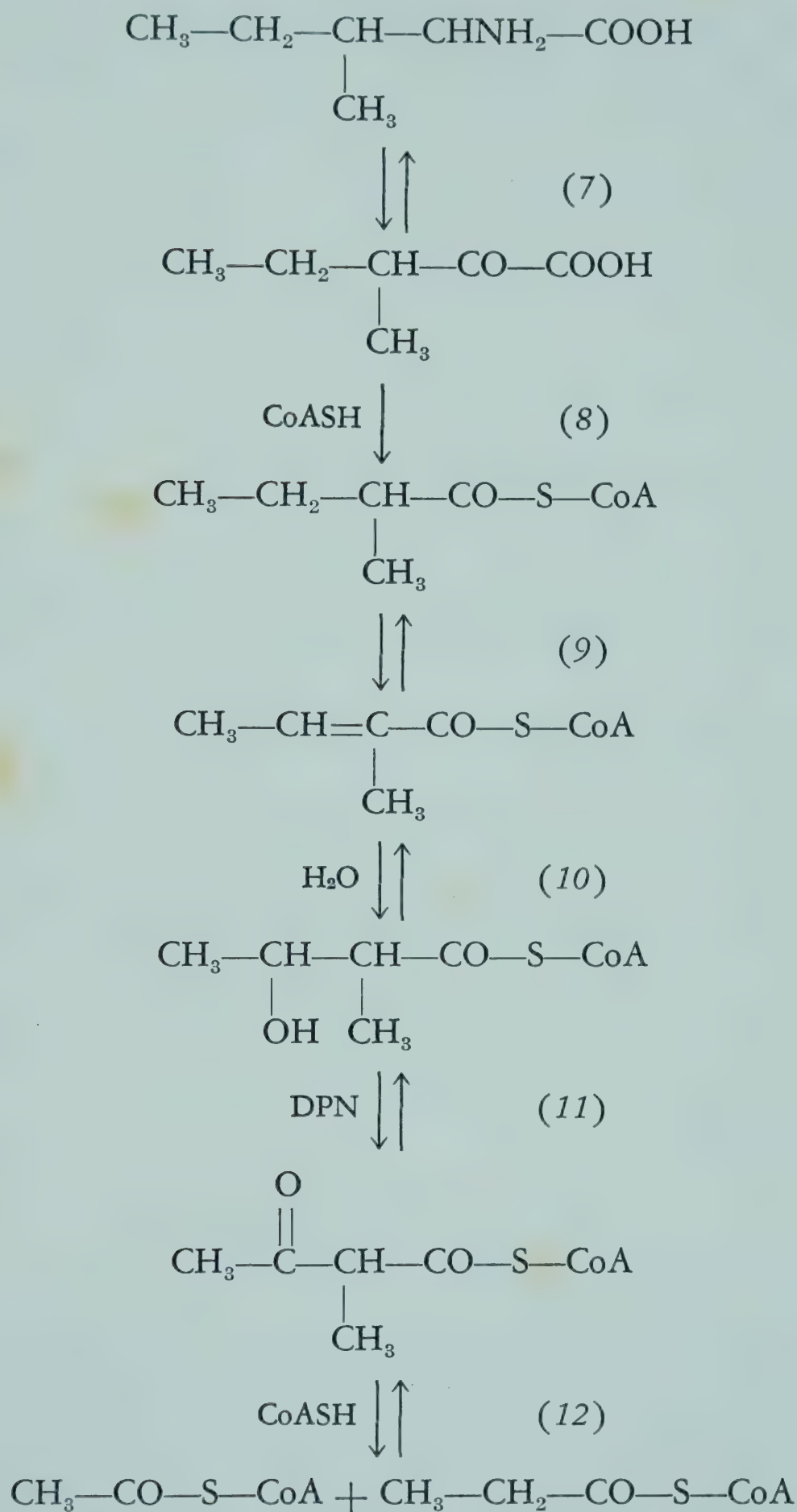


FIG. 2. Proposed scheme of isoleucine metabolism.

resulting thiol esters were converted to the hydroxamic acids. Chromatography in water-saturated butanol demonstrated the pres-



TABLE 3  
ENZYMATIC SYNTHESIS OF CITRATE FROM TIGLYL-CoA

Enzyme fraction added	DPN added $\mu M$ .	Citrate formed $\mu M$ .
Extract of pig heart (7.0 mg. protein)	2.0	0.26
Extract of rat liver (31.2 mg. protein)	2.0	0.33
Ammonium sulfate precipitate of rat liver extract (45.0 mg. protein)	2.0	0.41
Ammonium sulfate precipitate of rat liver extract (45.0 mg. protein)	None	0.06

The test system contained 500  $\mu M$ . Tris buffer, pH 8.1, 20  $\mu M$ .  $MgCl_2$ , 26  $\mu M$ . cysteine, 125  $\mu M$ . oxalacetate, 0.25  $\mu M$ . CoA, 1  $\mu M$ . tiglyl-CoA, and dialyzed enzyme preparations as indicated; volume 3.6 ml., incubation 60 minutes at 38° C.

ence of propionohydroxamic acid ( $R_f$  0.61) in addition to the  $\alpha$ -methyl- $\beta$ -hydroxybutyrylhydroxamic acid previously described. Neither of these spots exhibited ultraviolet absorption, unlike tigloylhydroxamic acid. Control experiments demonstrated that propionyl-CoA is not further metabolized in the enzyme preparation employed and that no acetyl-CoA accumulates in the presence of oxalacetate. The glycogenic and weakly ketogenic properties of isoleucine are readily accounted for by the formation of propionyl-CoA and acetyl-CoA, respectively.

#### VALINE METABOLISM

It has been well established that 3 of the 5 carbon atoms of valine and  $\alpha$ -ketoisovalerate (33) and of isobutyrate, the presumed degradation product (29), furnish glucose in the phlorhizinized dog. The amino acid has also been shown to yield small but significant amounts of glycogen in the fasting rat (8). More recent isotopic studies by Fones et al. (18, 26) and by Gray et al. (19) have provided additional information on this metabolic pathway, and Rose (31) has obtained isotopic evidence demonstrating that the isopropyl group of valine (and isobutyrate) is the source of the 3-carbon

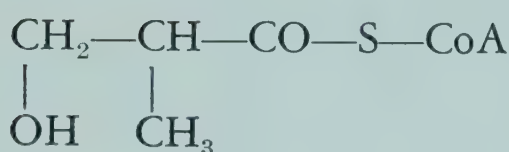
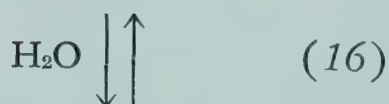
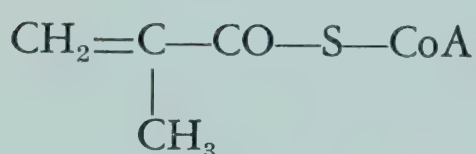
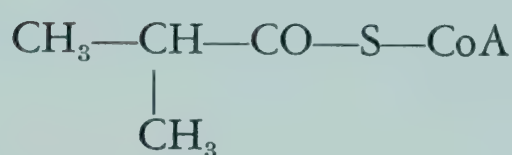
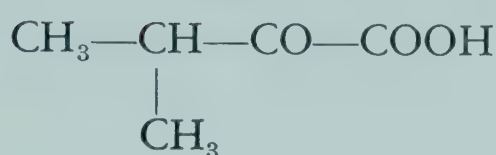


FIG. 3. Proposed scheme of valine metabolism.

fragment which is converted to glycogen and other tissue components. This finding supports the view that the branched-chain amino acids do not undergo demethylation and is consistent with the reactions proposed by Atchley (1) for the conversion of isobutyrate to propionate in washed kidney homogenates. It seems likely, however, that the sequence of intermediates (1) should be revised in accord with Reactions 13 to 16 (Fig. 3).

Methacrylyl-CoA ( $R_f$  0.58 in ethanol-acetate) was found to under-



go hydration in our heart enzyme system (Reaction 16) to furnish a new thiol ester ( $R_f$  0.81), presumably  $\beta$ -hydroxyisobutyryl-CoA. The corresponding hydroxamates were also separated on paper, and in support of the proposed hydration it was found that the new hydroxamic acid, unlike methacrylohydroxamic acid, exhibits no ultraviolet absorption. The interpretation of these results and of the apparent hydration of methacrylyl-CoA by crystalline liver crotonase (37) is complicated by the observation that methacrylyl-CoA undergoes spontaneous hydration to some extent at 38° C. The role of CoA in the additional reactions proposed by Atchley is unknown at the present time.

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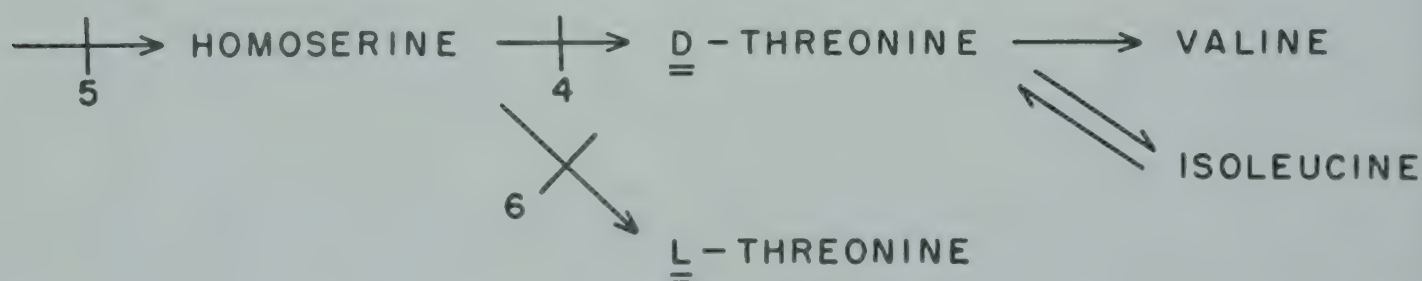
# SOME OBSERVATIONS ON THE BIOSYNTHETIC PATHWAY OF ISOLEUCINE \*

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A FEW YEARS AGO, a tentative scheme for the pathway of biosynthesis of isoleucine and valine was suggested in collaboration with Adelberg (14). This scheme was based mainly on results of qualitative growth experiments with mutant strains of several microorganisms and the isotopic data obtained by Tatum and Adelberg (12). The scheme, as then suggested, has since served as a working hypothesis for both laboratories, and for some time now it has been evident that several modifications are necessary.

The essential feature of the original proposal consisted of the idea that a compound, later shown to be homoserine, was the precursor of both valine and isoleucine and, in addition of L-threonine, which was believed to lie off the main pathway in the conversion of homoserine to valine and isoleucine:



More recently, quantitative growth experiments were reported (13) which suggested two major changes; that L-threonine was probably a more direct precursor of isoleucine than originally visualized, and that valine and isoleucine did not have a common precursor. It is the purpose of this discussion to summarize the recent evidence

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obtained in this laboratory from growth experiments and to consider the reports of other workers in order to suggest a scheme for isoleucine biosynthesis that seems more reasonable at this time.

The first evidence that valine did not arise from the same carbon chain as isoleucine came from growth studies with three kinds of threonineless mutants of *Escherichia coli* (see Table 1), namely, one

TABLE 1  
ISOLEUCINELESS MUTANTS OF *E. coli*, STRAIN K-12

Class	Designation	Compound utilized for growth
1	11A16	Isoleucine (valine stimulates)
2	20A19	Both $\alpha$ -keto- $\beta$ -methylvaleric and $\alpha$ -keto-isovaleric acids required
3	K-12 (inhibited by valine)	$\alpha,\beta$ -dihydroxy- $\beta$ -methylvaleric acid
4	JHM544	D-threonine, $\alpha$ -amino butyric acid
5	RSS-60	Homoserine, L-threonine, and, to a limited extent, D-threonine
6	12B14	L-threonine
4 and 6	JHM544B5	L-threonine and either D-threonine or $\alpha$ -amino butyric acid

utilizing D-threonine (blocked at step 4), one utilizing L-threonine (blocked at step 6), and one which could utilize either isomer as well as homoserine (blocked at step 5). The growth requirement of the D-threonine-requiring strain could be replaced entirely by isoleucine. It was believed that, in this instance, part of the isoleucine was broken down to an intermediate compound and served thus as a source of valine. However, it was observed that valine did not have any sparing action on isoleucine as had been predicted. Furthermore, isoleucine markedly spared the L-threonine requirement of mutants of class 5 and class 6 (Table 1), but the sparing effect was not improved by valine.

A second implication of the results of the experiments on the sparing action of isoleucine and valine was that L-threonine itself was probably directly on the biosynthetic pathway of isoleucine. This was demonstrated clearly by comparing the growth response of the D-threonine- or isoleucine-requiring mutant of *E. coli*, strain



JHM544, the L-threonine-requiring mutant, strain 12B14, and a third mutant, strain JHM544B5, blocked at both of the two loci. As is shown in Fig. 1, the isoleucine requirement was unaltered as a result of the second block. However, it can be seen that as a result of the second block in isoleucine synthesis, strain JHM544B5 responded

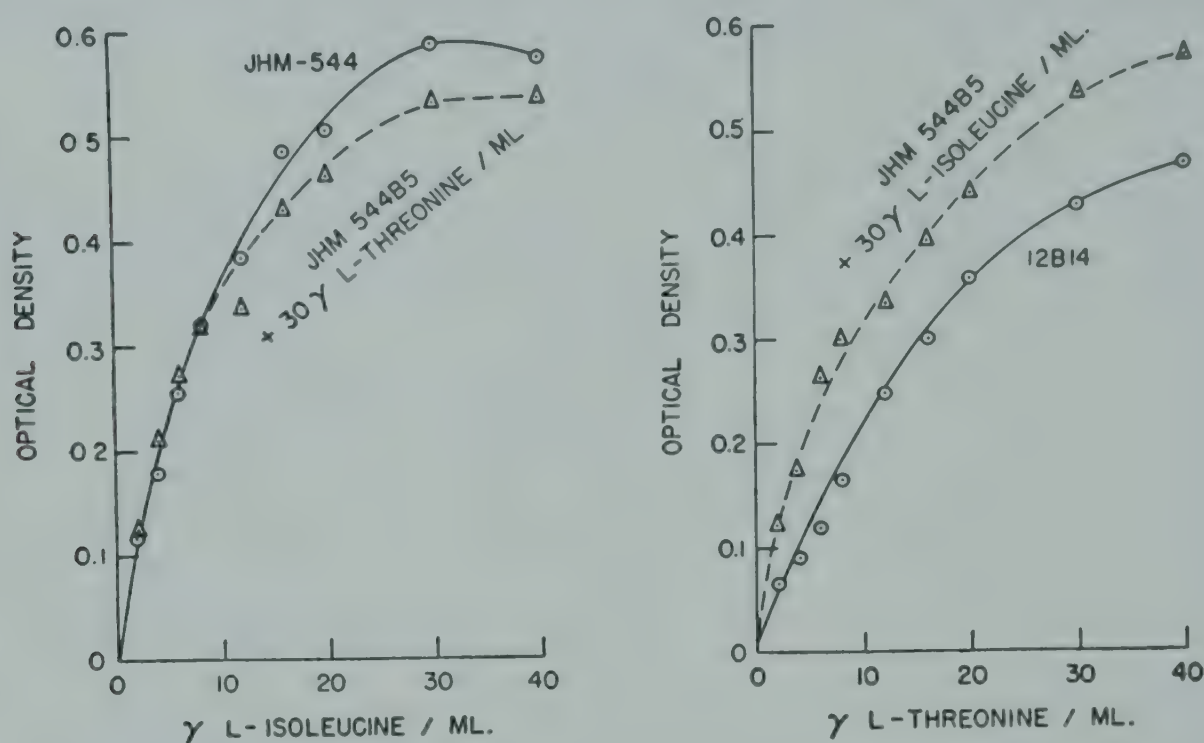


FIG. 1. The influence of a block in isoleucine synthesis on the L-threonine requirement in *E. coli*. Strain JHM544, isoleucineless, blocked at step 4. Strain 12B14, threonineless, blocked at step 6. Strain JHM544B5, doubly blocked at steps 4 and 6. Flask cultures shaken for 24 hours at 37° C.

more efficiently to L-threonine than does strain 12B14. It would appear that about one-half of the L-threonine used by strain 12B14 was diverted toward isoleucine synthesis. In the scheme shown in Fig. 2, L-threonine is therefore represented as a direct precursor of isoleucine.

The results of these growth studies are in agreement with findings of Abelson (1) who observed that L-threonine as well as  $\alpha$ -amino-butyric acid depressed the utilization of glucose carbon for isoleucine synthesis by *E. coli*. The existence of mutants of other microorganisms (14) similar to those mentioned here suggests that this relationship of L-threonine to isoleucine may be universal. Garner and Teas, for example, have reported that L-threonine carbon can be traced into isoleucine in *Neurospora crassa* (7).

Initially, there was no evidence as to which of the several four-

carbon compounds which strain JHM544 could utilize for growth was the earliest in the reaction chain, or, indeed, whether some were only indirectly related to isoleucine biosynthesis. Experiments with

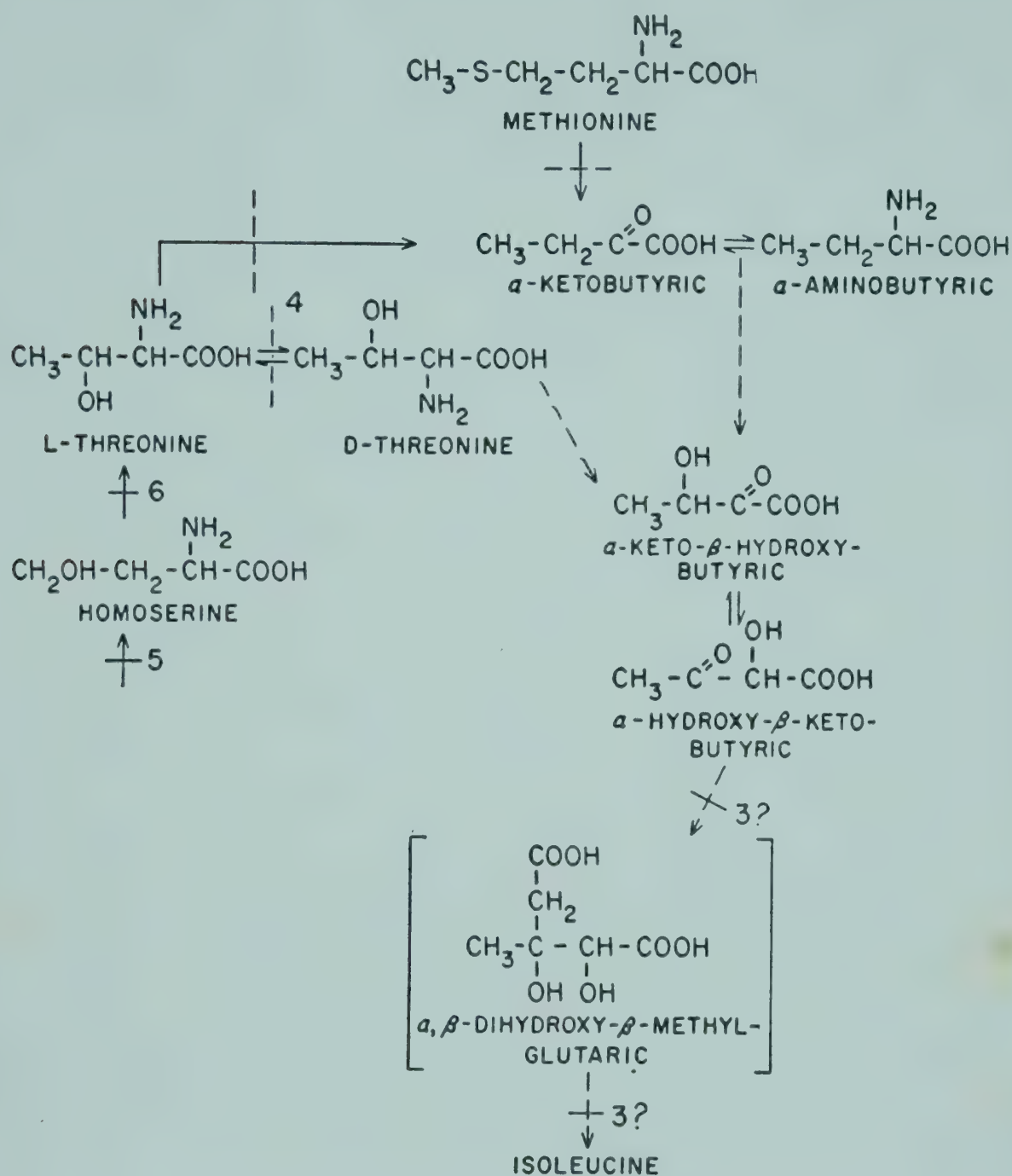


FIG. 2. Proposed biosynthetic pathway of isoleucine; the four carbon precursors of isoleucine. Solid arrows: steps for which some evidence exists. Broken arrows: steps for which only indirect evidence exists. Solid lines: site of genetically blocked reactions. Broken lines: metabolic blocks of unknown nature preventing function of known reactions for the biosynthesis of isoleucine.

D-threonine performed by Dr. Harold Amos at Harvard Medical School have demonstrated an enzymatic interconversion of L-threonine and D-threonine. The possession of this enzyme should enable the organism to grow equally well on the two threonine isomers. The mutants of class 4, which can utilize all of the four-carbon



compounds with the exception of L-threonine and homoserine, would consequently be presumed not to possess this threonine racemase. However, preliminary experiments have revealed the presence of threonine racemase activity in mutants of class 4. Obviously the enzyme is not sufficiently active in intact growing cells to permit them to convert the L-threonine to D-threonine in amounts required for growth. In very loose terminology, the limitation on the activity of this enzyme might be referred to as a metabolic block and, therefore, the reaction is interrupted by a broken line in Fig. 2.

A similar situation exists in regard to the role of the  $\alpha$ -ketobutyric acid. Mutants blocked at step 4 in the scheme utilize  $\alpha$ -ketobutyric acid as a substitute for isoleucine (or D-threonine). The reason why mutants of this class are unable to utilize L-threonine by the conversion of the latter to  $\alpha$ -ketobutyrate remains unexplained, since L-threonine deaminase activity (16) can be demonstrated in cells of this type. There would appear to be some internal metabolic block (indicated again by a broken line) preventing this reaction from proceeding at a rate sufficient to permit L-threonine to serve as a growth factor for this mutant. Another possible alternative enzyme system for the formation of  $\alpha$ -ketobutyrate (and, hence, of isoleucine) is the methionine dethiomethylase described by Kallio (8), which converts methionine to  $\alpha$ -ketobutyrate. Again, since methionine will not permit growth of mutants of class 4, or even exert a sparing effect on the threonine requirement of strain 12B14 (class 6), we can only guess that the rate of this reaction under conditions of growth is insufficient.

Information as to how  $\alpha$ -ketobutyric acid can be converted to any compound thought to be on the pathway to isoleucine has not been obtained. For this reason its entrance into the pathway has been indicated by a broken arrow in Fig. 2. The same holds true for  $\alpha$ -aminobutyric acid, since the two compounds are readily interconvertible by a transaminase.

In view of the above observations it is not possible at this time to establish the genetic block in class 4 mutants. Nevertheless, without regard to the relationship of D-threonine or  $\alpha$ -ketobutyric acid



to the pathway of isoleucine synthesis, the other four-carbon compound utilized by strain JHM544,  $\alpha$ -keto- $\beta$ -hydroxybutyric acid, seems to be the most logical compound to be next in the pathway, since its tautomer,  $\alpha$ -hydroxy- $\beta$ -keto-butyrac acid might serve as an acetyl acceptor in the formation of the postulated intermediate  $\alpha,\beta$ -dihydroxy- $\beta$ -methylglutaric acid. However, as yet no transaminase or dehydrogenase has been found which will convert D-threonine to its  $\alpha$ -ketoanalog.

In this regard a paradox should be mentioned. D-Threonine serves as a growth factor for strain JHM544 only under highly aerobic conditions. Similarly, its sparing action on the L-threonine requirement or homoserine requirement of strain RSS-60 (blocked at step 5), one of the steps between aspartic acid and homoserine (5, 9), is exhibited only under aerobic conditions. Attempts to find an oxidative reaction involving D-threonine have led somewhat astray in that the oxidation of D-threonine proceeds much too far to account for its conversion to the corresponding  $\alpha$ -keto-acid. The oxidative destruction of D-threonine probably accounts for extremely inefficient utilization of it in media containing glutamic acid and alanine as carbon sources, as well as the somewhat inefficient utilization in glucose medium that has been observed. Nevertheless, under aerobic conditions, when D-threonine would not be oxidatively destroyed, it cannot serve as a growth factor.

Another example where internal inhibition probably affects the growth response of these mutants is noted when strain 12B14 (class 6) and strain RSS-60 (class 5) are compared. Mutants of the latter class grow well when given either L-threonine or homoserine and use isoleucine, D-threonine, and  $\alpha$ -ketobutyrate to a limited extent. Strain 12B14 responds only to L-threonine and even though, like strain RSS-60, it has ability to convert D-threonine to L-threonine, it cannot utilize even for limited growth D-threonine or the other compounds on that side of step 4.

The compound shown in brackets,  $\alpha,\beta$ -dihydroxy- $\beta$ -methylglutaric acid, has not yet been shown to be able to substitute for isoleucine in strain JHM544 (class 4). The inactivity of the synthetic material



may have been due to the fact that the wrong environmental condition has been employed, so that the compound had not entered the cell.

It is not until we proceed beyond the questionable compound in brackets that we come to a compound which has a clearer role in the

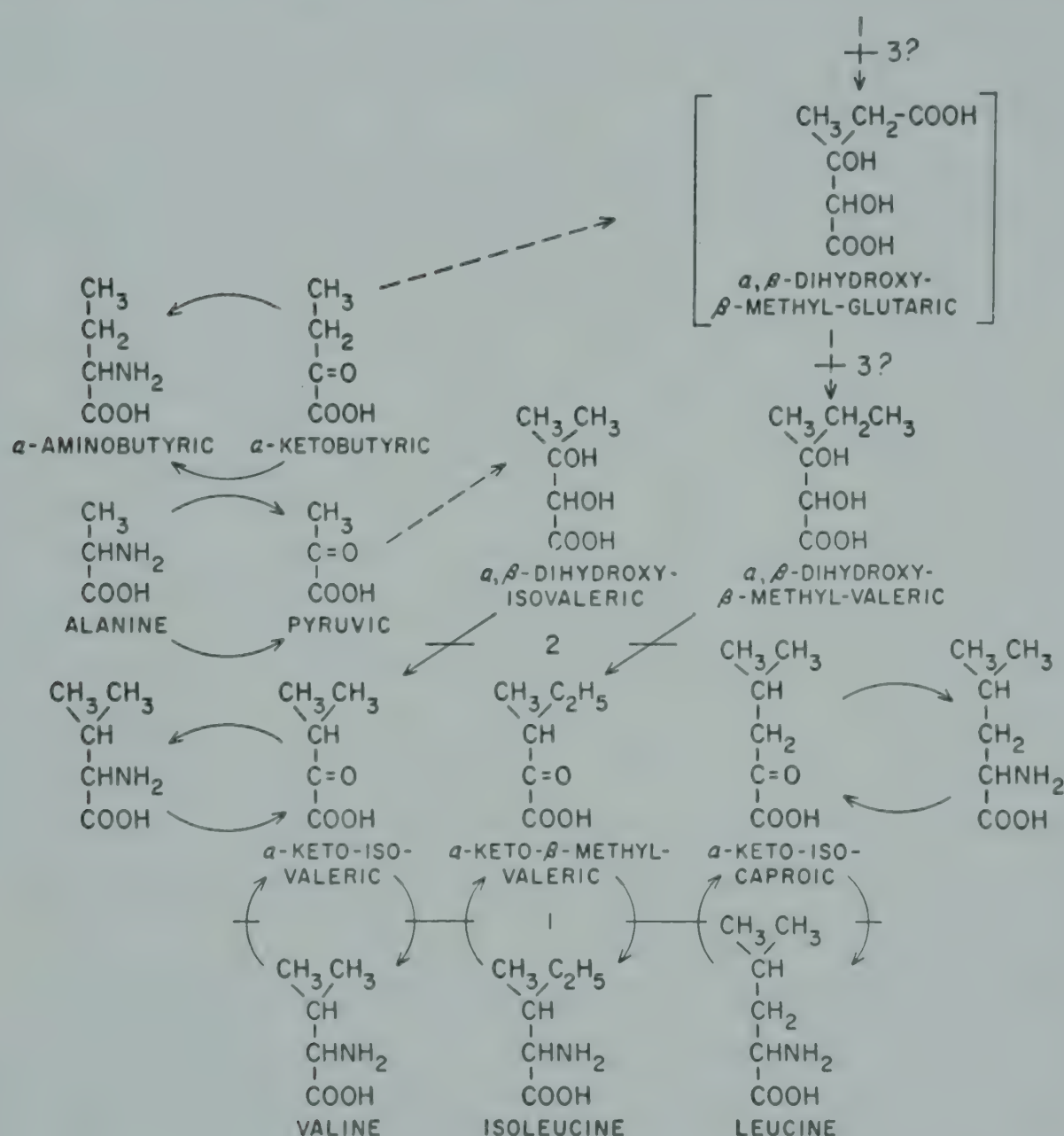


FIG. 3. Proposed biosynthetic pathway of isoleucine; the six-carbon precursors. Symbols as in Fig. 2.

pathway to isoleucine (see Fig. 3). This is the isoleucine precursor,  $\alpha, \beta$ -dihydroxy- $\beta$ -methylvaleric acid (2) isolated by Adelberg along with the corresponding valine precursor  $\alpha, \beta$ -dihydroxyisovaleric acid (3). From this point on, the reactions leading to isoleucine and valine parallel each other and the compounds retain their structural similarity. This structural similarity which is maintained

in the final stages in the biosynthesis of these two compounds is probably of considerable importance as a basis for the competitive interactions which have been observed between these two compounds (6, 15).

Mutants blocked in the next step in the biosynthesis of isoleucine (step 2) require not only isoleucine for growth but also valine. This double deficiency implies that the enzyme which is missing in mutants of this class catalyzes the removal of water from both dihydroxy acids to form the immediate precursors of isoleucine and valine, the corresponding  $\alpha$ -keto acids.

The final step in the biosynthesis of isoleucine must be considered in relation to three enzyme activities separated from *E. coli* extracts by Rudman and Meister (10). Transaminase B, which transfers the amino group of glutamic acid not only to  $\alpha$ -keto- $\beta$ -methylvaleric acid but also to  $\alpha$ -ketoisovaleric and  $\alpha$ -ketoisocaproic acids, is missing in *E. coli* mutant 11A16 (step 1 in Fig. 3). Transaminase A, which transfers amino groups to and from leucine and several other amino acids, including glutamic acid but not isoleucine, is represented at the right of Fig. 3. The three amino acids reacting with the valine-alanine transaminase are shown at the left of Fig. 3. The relative roles of the latter enzyme and of transaminase B have been adequately explained by Rudman and Meister (10).

Recent experiments with *E. coli* strain K-12 and mutant 11A16 (lacking transaminase B) suggests that  $\alpha$ -ketoisocaproic acid amination is a relatively superfluous property of transaminase B in the economy of the growing cell (11). Strain 11A16 has only about one-third the leucine-glutamic acid transaminase activity of the wild strain due to the absence of transaminase B. Apparently, this strain can supply its whole quota of leucine by means of transaminase A, since the growth of this mutant is the same in the presence and absence of leucine. In contrast, valine markedly stimulates the growth of strain 11A16 indicating that the activity of the valine-alanine transaminase is inadequate for supplying valine for growth. Further, cells of the wild strain grown in the presence of isoleucine



were found to contain about 20 per cent less transaminase B activity than did cells grown in minimal medium. The addition of valine and isoleucine to the medium resulted in a decrease of about 40 per cent in transaminase B activity. The addition of leucine to the minimal medium either alone or in combination with isoleucine and valine is without effect on the activity of transaminase B in the cells. Thus it seems that transaminase B in the growing cell is essential for isoleucine formation, necessary for maximal valine formation, but dispensable for the formation of leucine.

A feature of the biosynthetic pathway of isoleucine that is revealed in Fig. 3 is the series of interactions between valine and isoleucine. Thus, the two final steps in the synthesis of each are catalyzed by common enzymes. Further, valine itself will exchange its amino group with an isoleucine precursor ( $\alpha$ -keto-butyrate) and with pyruvate, which, according to Abelson's data (1) is in some way a precursor of valine itself (this transformation being indicated by a broken arrow in Fig. 3). These interactions undoubtedly underlie the rather narrow limits in the relative amounts of valine and isoleucine which permit the growth of many microorganisms, notably the K-12 strain of *E. coli*. In the case of strain K-12, the balance between isoleucine and valine is probably shifted to the extreme in favor of the latter, since its growth is so sensitive to exogenous valine. Evidence that in this strain the step which makes isoleucine a limiting metabolite may lie as far back as homoserine formation is the observation that all of the compounds suggested in Figs. 2 and 3 as precursors of isoleucine are stimulatory to the growth of strain K-12 in minimal medium.

ADDENDUM — The data presented in this symposium both by Adelberg and Weinhouse prove that  $\alpha,\beta$ -dihydroxy- $\beta$ -methylvaleric acid is not an intermediate in isoleucine biosynthesis. At present their data do not permit any decision to be made as to which four-carbon compound participates in the condensation reaction. Their observations do not, however, affect any other aspects of the scheme presented in Figs. 2 and 3.

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# ISOTOPE STUDIES ON BIOSYNTHESIS OF VALINE AND ISOLEUCINE<sup>1</sup>

MURRAY STRASSMAN<sup>2</sup> and SIDNEY WEINHOUSE

As ADELBERG has pointed out, we suggested that in the biosynthesis of the valine carbon chain in yeast there occurs an intramolecular migration of a methyl carbon (1, 2). The data on which this conclusion was based, along with other data which led us to extend this suggestion to the biosynthesis of the carbon skeleton of isoleucine, will now be presented.

The experimental procedure employed in these studies was to grow *Torulopsis utilis* on glucose as essentially the sole carbon source, together with tracer quantities of various metabolic intermediates labeled with carbon-14. The yeast cells were harvested, and, after hydrolysis of the proteins, the individual amino acids were isolated by chromatography on Dowex columns. The amino acids thus isolated in pure form were then degraded to obtain individual specific activities of each carbon atom. It was found that neither the carboxyl nor methyl carbons of acetate were readily incorporated into valine, whereas all three lactate carbons were highly incorporated. Data on the incorporation of lactate carbons are shown in Table 1. It is evident that the carboxyl carbon of lactic acid appears virtually exclusively in carbon 1, the carboxyl carbon of valine; the  $\alpha$  carbon of lactate appears almost exclusively and to equal extent in carbons 2 and 3 of valine; and that the methyl or  $\beta$  carbon of lactate is incorporated to a high degree in only the valine methyl carbons. Since these two are equivalent, a separate assay cannot be obtained on these two carbons. The last column of this table shows

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TABLE 1

DISTRIBUTIONS OF LACTATE AND GLUCOSE CARBONS IN VALINE

Valine Carbon Number	CH <sub>3</sub>	Lactate CHOH	COOH	Glucose Carbon 1
1	1	3	99	3
2	4	49	0	6
3	4	47	} 1	6
4,4'	91	1		85

that carbon 1 of glucose appears preponderantly in the methyl carbons of valine. This finding is in accord with expectations based on the well-established mechanism of glucose catabolism via the Embden-Meyerhof process, according to which carbons 1 and 6 of glucose form carbon 3 of lactate. This experiment with glucose-1-C<sup>14</sup> clearly indicated also that both methyl groups of valine arise from the methyl carbon of lactate, since the specific activity of this position was approximately 40 per cent of that of the glucose 1 carbon employed as the substrate. If only one methyl group was labeled, the specific activity of the valine methyl carbon could not be more than one-fourth that of glucose 1 carbon.

The equal labeling of carbons 2 and 3 of valine from the lactate  $\alpha$  carbon was regarded as a key observation suggesting that, in the formation of the valine carbon skeleton, two  $\alpha$ -carbons of lactate undergo coupling. A conceivable biological reaction fulfilling these requirements appeared to be the well-known ketol condensation of pyruvate and acetaldehyde to yield acetolactic acid, as shown in Fig. 1 (2).

According to this formulation, the formation of the valine carbon chain takes place by migration of the methyl group of the pyruvate moiety to carbon 2 of the acetaldehyde moiety. This type of molecular rearrangement is commonly observed with ditertiary glycols such as pinacol, and such a reaction with acetolactic acid to yield the keto analog of valine seems highly plausible.

Once a reaction of this type was conceived, it was evident that the biosynthesis of the isoleucine carbon chain might proceed by an analogous series of reactions, in which acetaldehyde, derived from pyruvic acid, might condense with  $\alpha$ -ketobutyric acid.



Adelberg has already pointed out that  $\alpha$ -aminobutyric, and hence  $\alpha$ -ketobutyric, acid very likely originates from aspartic acid, via homoserine and threonine. On the assumption that aspartic acid, derived from 4-carbon acids of the citric acid cycle, provides the carbon chain of  $\alpha$ -ketobutyrate, it is possible to calculate a theoretical

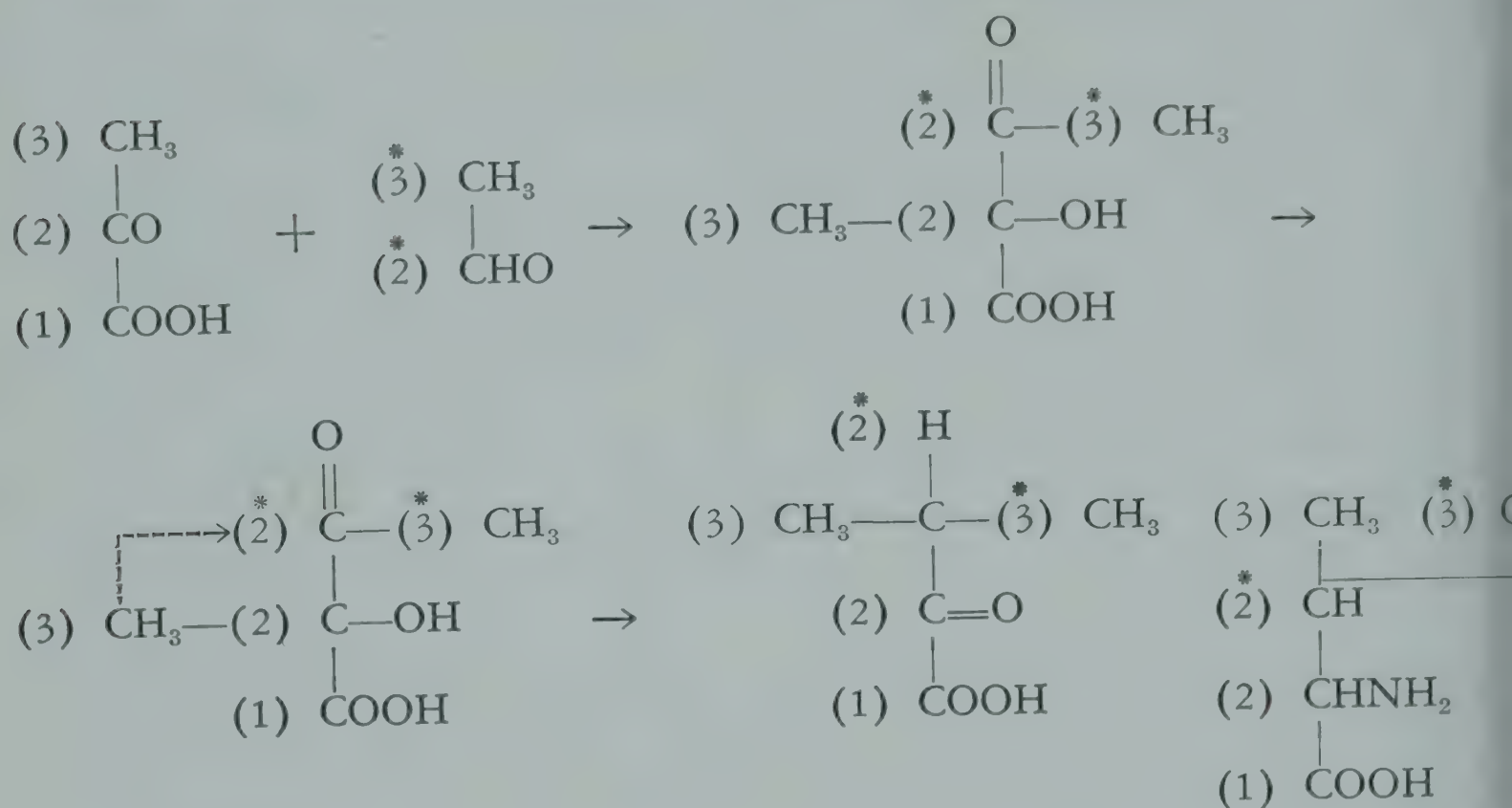


FIG. 1. Postulated Mechanism of Valine Biosynthesis.

distribution of acetate methyl and carboxyl carbons in the carbon chain of isoleucine. Aspartic acid derived from the citric acid cycle would have methyl carbons distributed equally in its  $\alpha$  and  $\beta$  carbons, with twice the activities in these positions as in the  $\alpha$  and  $\gamma$  carboxyl carbons. Acetate carboxyl carbons would appear exclusively and equally in the aspartate carboxyl carbons. On the basis of the series of reactions shown in Fig. 2, we would expect carbons 1 and 2 of isoleucine to represent aspartate carbons 1 and 2 respectively, carbons 4 and 5 would represent aspartate carbons 3 and 4 respectively, and we would expect carbons 3 and 6 to represent pyruvate carbons 2 and 3 respectively. It is thus possible to calculate the theoretical distribution of acetate methyl and carboxyl carbons in isoleucine, and these are portrayed in Table 2.

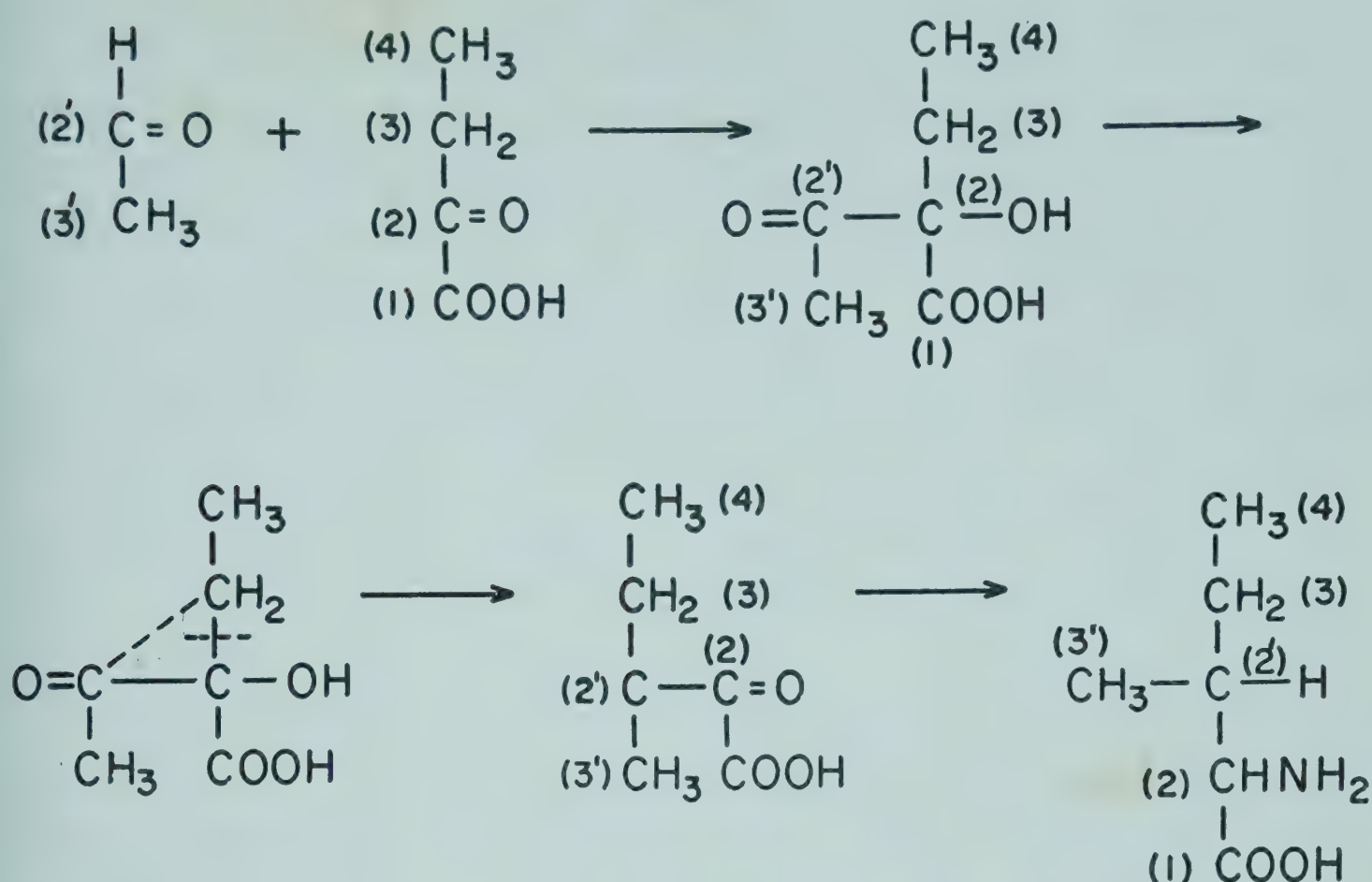


FIG. 2. Postulated Mechanism of Isoleucine Biosynthesis from Acetaldehyde and  $\alpha$ -Ketobutyrate.

TABLE 2

OBSERVED  $\text{C}^{14}$ -DISTRIBUTIONS AND VALUES CALCULATED ON BASIS OF CONDENSATION OF ACETALDEHYDE WITH  $\alpha$ -KETOBUTYRIC ACID

Isoleucine Carbon	Acetate Methyl		Acetate COOH		Lactate Methyl	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
1	17	17	47	50	5	Low
2	39	33	1	0	15	Intermediate
3	4	0	3	0	5	Low
4	19	33	0	0	27	Intermediate
5	18	17	46	50	7	Low
6 *	3	0	3	0	41	High

\*  $\beta$ -methyl group.

Alongside each column of calculated values are given the observed values. The agreement between these was very gratifying. In every carbon atom except number 4, the agreement was extremely close. We are not yet certain why this carbon atom has a lower value than



the calculated one; the degradation is being repeated. However, the remarkable similarity in the over-all distribution pattern leaves no doubt in our minds as to the essential correctness of the mechanism shown in Fig. 2. This is also emphasized by comparing the observed and calculated distributions of the acetate carboxyl carbon. In only carbons 1 and 5, namely, those which represent carboxyl carbons of aspartate, was there observed any significant activity. Elsewhere the carbons were devoid of activity, as anticipated from the mechanism described. Degradations of isoleucines derived from experiments with the labeled lactates are not yet completed, and full data are available only for  $\beta$ -labeled lactate. It is obvious from the last two columns of Table 2, however, that the distribution of lactate methyl carbon is in complete accord with the mechanism proposed. Since lactate not only provides acetaldehyde but also acetate, it is impossible to make the type of calculation carried out for the acetate carbons. However, we can make certain predictions concerning the distribution of lactate carbons in isoleucine. Since lactate  $\beta$  carbon should yield methyl-labeled acetate, we should expect the distribution to be similar to that of acetate methyl; but since it is also the source of the methyl carbon of acetaldehyde, we should expect highest activity in carbon 6 of isoleucine. To summarize, we should expect high activity in carbon 6, intermediate activities in carbons 2 and 4, and low activities in carbons 1, 3, and 5 of isoleucine. That these are the observed distributions constitutes further evidence of the essential correctness of the mechanism shown in Fig. 2.

It seems inconceivable that the close agreement between observed and theoretical distribution patterns could be a result of coincidence, but it must be kept in mind that the isotope data do not decisively identify intermediates. For this purpose, enzyme studies or investigations with mutants appear to be the next phase in the testing of this hypothesis.

Although intramolecular migrations of the type required by this formulation have not yet been observed in biological systems, the reaction is quite plausible from an organic chemical standpoint.



Recent studies of cholesterol synthesis (3) suggest that in the cyclization of squalene, methyl group migrations may also occur. It is conceivable that alkyl group migrations may play an important role generally in the biosynthesis of branched carbon chains.

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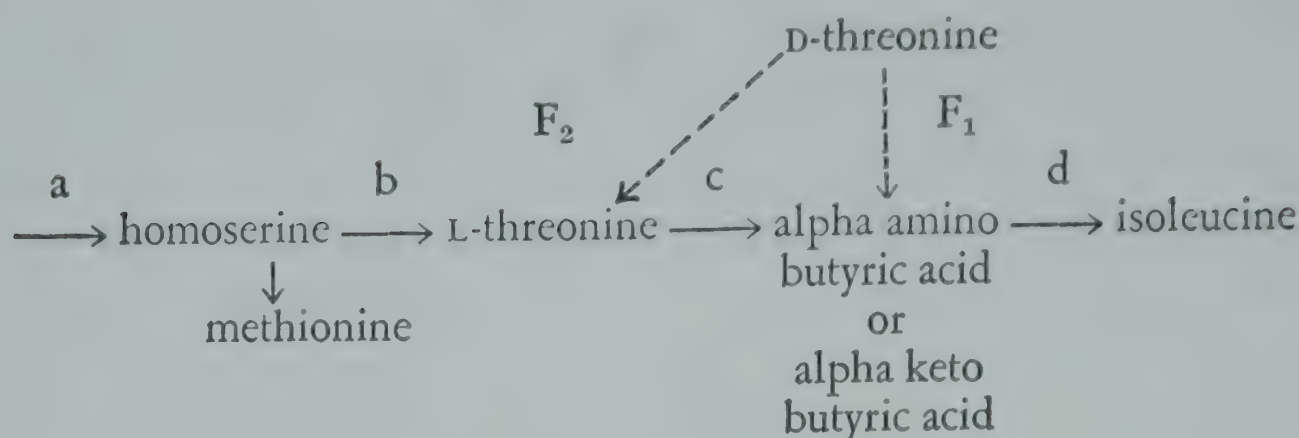
## DISCUSSION

DR. DAVIS: Yesterday, Dr. Roberts told us that his group showed that in *E. coli*, growing on glucose, labelled threonine went very well into 4 of the carbons of isoleucine. It was therefore concluded that L-threonine was indeed on the path from glucose to isoleucine. Dr. Umbarger and Dr. Adelberg indicated to me that they favored the same interpretation. I would like to present some information on 4 mutants of *E. coli* that has led us to consider this interpretation unlikely.

Mutant 1 is the type that Dr. Umbarger mentioned that requires L-threonine; it shows no response whatsoever to any of the other compounds mentioned. Mutant 2 is a frequent mutant of the W strain of *E. coli*, it requires isoleucine, and responds about equally well to D- or L-alpha amino butyric acid or to alpha keto butyric acid. It responds somewhat more slowly to D-threonine. L-Threonine not only is not used, but it effectively competes with the utilization of D-threonine (but not with that of the other growth factors mentioned). Mutant 3 has been obtained from Dr. Lederberg, and is derived from a different strain of *E. coli* (K-12); it grows on all of the compounds that are active for mutant 2, and will also grow, though quite slowly, on L-threonine. Mutant 4 is blocked before homoserine. It is well known, since the work of Horowitz, that with such mutants of *Neurospora* homoserine can be replaced by methionine plus L-threonine. In our experience this was also true with such mutants in *E. coli*, but the growth on L-threonine plus methionine was slow; it could be restored to normal rate by the further addition of isoleucine or alpha amino butyric acid or alpha keto butyric acid.

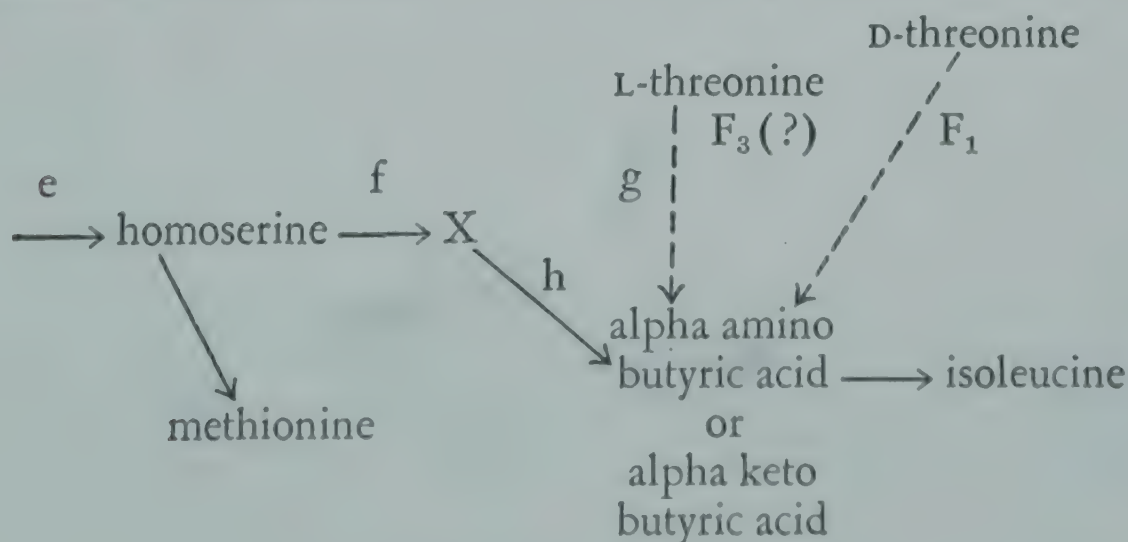
I think it would be difficult to account for these facts in any scheme, such as the following, where L-threonine is placed on the normal path, via alpha amino or alpha keto butyric acid, to isoleucine.





Mutant 4, which responds to homoserine, would presumably be blocked in position a; but the reason that isoleucine accelerates the growth of this strain on methionine plus even an excess of threonine would not be evident. Mutant 1, which responds only to L-threonine, would presumably be blocked in position b. The excellent response to L-threonine would imply rapid penetration of this substance, and the failure to respond to alpha amino butyric acid or isoleucine would imply that the path from L-threonine to alpha amino butyric acid is irreversible. It would then be difficult to explain mutant 3, which responds well to isoleucine and poorly to L-threonine. This strain could not very well be blocked at b, because of the properties just noted for mutant 1; and if it were blocked at c, one would have to assume an incomplete block which could be "forced through" by an excess of its substrate. While such a mechanism is conceivable, I know of no precedent for it. Finally, mutant 2 would presumably be blocked in position c, and would present no special problem beyond the necessity of accounting for its response to D-threonine. Since no one has suggested that this compound is directly on the normal path to isoleucine, I believe we would all be in agreement in assuming some side reaction,  $F_1$  or  $F_2$ , for converting D-threonine to a member of that path.

While each of the difficulties raised by this scheme can be met by appropriate *ad hoc* assumptions, the variety of these difficulties makes me skeptical about this scheme. In contrast, these findings can be readily explained by the following scheme (or a modified one in which L-threonine would arise from homoserine directly rather than from the postulated intermediate X).



Here mutant 4 would be blocked at e, and its relative requirement for isoleucine, in addition to its absolute requirements for threonine and methionine, would be explained. Mutant 1 would be blocked at g; its lack of response to compounds other than L-threonine would not, in contrast to the previous scheme, imply whether or not the path to isoleucine is reversible. Mutant 2 would be blocked at h; its response to D-threonine would depend, as in the previous scheme, on facultative reaction  $F_1$ . Its lack of response to L-threonine would imply absence in this organism of a similar facultative reaction ( $F_3$ ) for converting this compound to alpha amino butyric acid. Finally, mutant 3, which can slowly utilize L-threonine, could also be blocked at h, and would differ from mutant 2 only in being derived from a wild type that had the possibility of developing reaction  $F_3$  in the presence of L-threonine. This interpretation is based on the expectation that the nature of the obligatory biosynthetic paths would not vary from one strain of *E. coli* to another, whereas it is known that strains can vary qualitatively in their ability to develop facultative, adaptive paths (e. g., those concerned with utilizing carbon sources). (Another possible interpretation for mutant 3 would be that it is blocked in reaction f; its good response to isoleucine and poor response to L-threonine would then depend on the relative reversibility of the two paths from compound X.)

It is obvious that this question has not been definitively answered by either the isotopic or the mutant-nutritional studies carried out so far; and as I shall emphasize in the talk on aromatic biosynthesis, I think the most rigorous proof available at present that a compound is a normal or obligatory intermediate in a microorganism has been furnished by enzymatic studies. Such studies on the present problem are now being successfully prosecuted by Georges Cohen and Ben Nisman in Paris. Meanwhile, the main point I should like to make here is that while isotopic studies offer the most rigorous possible evidence that a cell *can* use a given compound as a precursor, we are likely to be occasionally led astray if we draw the further conclusion that this compound necessarily *is* an intermediate in normal biosynthesis on minimal medium. This is an old story in nutritional studies. Thus Tatum and Beadle showed many years ago that *p*-nitrobenzoic acid could replace *p*-aminobenzoic acid as a growth factor. Yet few would consider *p*-nitrobenzoic acid a normal precursor of *p*-aminobenzoic acid, even if one used isotopes till the cows came home to prove that *p*-nitrobenzoic acid got incorporated into *p*-aminobenzoic acid.

One might argue that this analogy is not relevant since L-threonine is synthesized by the cell whereas *p*-nitrobenzoic acid presumably is not. However, a more closely parallel case is offered by the path from histidine to glutamic acid, whose beautiful recent analysis has been reviewed here by Dr. Tabor. Dr. Magasanik has shown that this path is inductive in some bacteria even though they can synthesize histidine. This means that the



endogenously synthesized histidine does not induce the development of this degradative path, or at least does not do so nearly as effectively as exogenous histidine.

In these remarks on the isotopic method, I have tried to emphasize only a limitation of its application to microorganisms. In animals it is possible to introduce a labelled compound in concentrations that do not significantly change its concentration in the body fluids, and this fact gives us confidence that isotopes can reveal the kinetics of reactions under strictly physiological conditions. With microorganisms, in contrast, the investigator has the burden of proving that the addition of a compound to a minimal medium, even at low concentration, has not produced a significant physiological change in the organism. In this connection one might call attention to the well-known ability of bacteria of many species to build up a high intracellular concentration of certain compounds when provided externally with these compounds at a low concentration. Furthermore, though we are accustomed with ordinary cultures to providing most amino acids at around 20  $\gamma$ /ml., this choice is dictated by the *amounts* necessary for an adequate amount of growth, rather than the *concentrations* necessary for an adequate rate of growth. There is an enormous difference between these two figures: Novick and Szilard have shown, in the steady-state conditions provided by the chemostat, that maximal growth rate of *E. coli* mutants is supported by the required amino acid at a concentration roughly 1/1000 of that just noted.

DR. VOGEL: I shall try to reply to this as briefly as possible. Substantial evidence is available for the following isoleucine path reported, for example, by Abelson and collaborators:

aspartic acid  $\rightarrow$  homoserine  $\rightarrow$  threonine  $\rightarrow$   $\alpha$ -ketobutyric acid  $\rightarrow$  isoleucine.

I would just like to make the point that the mere recitation of growth responses of mutants (which have repeatedly been described in the literature) can hardly be considered as supporting a scheme such as Dr. Davis advanced, since plausible alternative explanations have not been excluded. The first type of mutant mentioned (responding to L-threonine only) does not seem to have an immediate bearing on the specific question raised. Mutants of the second type which fail to respond to L-threonine may be blocked through some kind of deficiency in L-threonine dehydrase. Several possibilities, singly or in combination, must be considered in attempting to account for such a deficiency, among them, unusual sensitivity of this enzyme to inhibition by (the substrate) L-threonine or a chemical relative, disruption of the organization of this enzyme, or a suboptimal production of the enzyme, associated with internal inhibition effects. If this type of mutant indeed has a deficiency in L-threonine dehydrase, its behavior is in line with the pathway of Abelson et al. rather than with the scheme presented by Dr. Davis (since such a deficiency would account both for the requirement for L-isoleucine



or a related compound and for the lack of response to L-threonine). D-Threonine presumably works by giving rise to  $\alpha$ -ketobutyrate, either by the action of a D-threonine dehydrase or perhaps in a less direct manner. The mutants responding to L-threonine as well as to D-threonine (in addition to the other compounds mentioned) may well have a diminished ability to synthesize L-threonine. The latter amino acid would then be expected to promote growth, and isoleucine or its intermediates (and related compounds) would work through sparing of the limiting L-threonine. The behavior of the homoserine-requiring mutants appears to be partly due to inhibition phenomena, and is of unknown significance regarding the point in question.

The suggestion that exogenous L-threonine induces an adaptive, facultative system is rendered improbable by the isotope work which Dr. Davis referred to. The speed and extent of incorporation of either relatively large or minute amounts of tracer threonine into isoleucine suggest that the system converting threonine to isoleucine is present and functional at the time of addition of the tracer threonine; and although Dr. Davis says that one can do isotopic studies until the cows come home, I don't think that mutant studies are necessarily correct and that the other studies are necessarily incorrect.

DR. KNOX: There seem to be quite a number of arrows drawn which represent reactions which need enzymatic confirmation. We can call these "reactions in search of enzymes." Now, I have a modest store of enzymes which are in search of substrates. These are enzymes which catalyze types of reactions which have not been shown to be catalyzed by enzymes before, and I thought that one might fit in possibly at the urocanic acid stage. If you remember, Dr. Waelsch wrote an enol and a keto form of imidazole propionic acid. When this first tautomerization occurs, the configuration of one of the carbon atoms would change, but he is quite definite in saying that the product is an L-amidino glutaric acid, so that this tautomerism cannot be completely free. I would suggest that this enol to keto change could occur under the influence of an enzyme of the sort which we found recently in studying tyrosine metabolism, an enol-keto tautomerase. We call it that because the nicer name of enolase had been pre-empted some time ago for a different type of reaction. The reaction is simply the enol to keto change of phenylpyruvic acid, which we can follow by the very marked extinction of the enol. It has a molecular extinction coefficient of around 12,000 compared to the more or less zero extinction of the keto formed. We have not been able to prove that the enzyme forms from the keto form a *cis*- or a *trans*-enol, but assuming the 3-point attachment hypothesis of Ogston, the enzyme could save the configuration of this carbon atom during such a change. On the other hand, if urocanic acid itself, which as I remember has a double bond in the side-chain, works only as the *cis*- or the *trans*-urocanic acid, there is also an enzyme (which I will talk about Thursday) which catalyzes the *cis-trans* isomerization and which has as its coenzyme, glutathione.



# SOME COMPARATIVE ASPECTS OF LYSINE METABOLISM

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LYSINE PRESENTS an extreme example of the impossibility of extrapolating knowledge gained about the metabolic route taken by a substance in one type of organism to the pathway in another kind of creature. In view, however, of the many ways in which lysine metabolism differs from that of other amino acids, we should not let the impossibility of arguing from *Neurospora* to *Escherichia coli*, in this case, depress us unduly with regard to the metabolism of amino acids in general. We should rather take the view that this apparent lack of biochemical unity is just another peculiarity of lysine.

In this review, some comparative aspects of lysine metabolism will be described, and some attempt will be made to integrate much scattered and apparently contradictory information.

## RATS

The  $\alpha$ -amino group of lysine, like that of threonine, does not contribute to the metabolic pool of nitrogen in the rat; neither does it interchange with administered  $N^{15}$ , either in  $NH_3$  or in glycine (91, 19, 37). This suggests that lysine does not take part in the general reversible transamination of  $\alpha$ -keto acids, and no evidence for this has been obtained in vitro. However, Meister (57) has found that a rat liver glutamine transaminase preparation will transfer amino groups between glutamine and  $\epsilon$ -N-substituted  $\alpha$ -keto analogues of lysine, but not with the unsubstituted  $\alpha$ -keto acid.

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Meister also suggested that, by analogy with ornithine, the  $\delta$ -amino group of which will transaminate to form glutamic semialdehyde, the  $\epsilon$ -amino of lysine might also be active, yielding  $\alpha$ -amino adipic- $\epsilon$ -semialdehyde (see also page 468).

The rate of oxidative deamination of lysine by the L- and D-amino acid oxidases of mammalian kidney is extremely slow compared with that of other amino acids (8). Here again, the  $\epsilon$ -N-substituted derivatives of lysine were found by Neuberger and Sanger (61) to be more susceptible than lysine, and the suggestion was made that acetylation of the  $\epsilon$ -amino group might be a preliminary step in the degradation of lysine. So far no experimental evidence has been obtained in support of this idea, beyond the facts that  $\epsilon$ -N-substituted lysine derivatives are more susceptible to transamination and deamination (see also 55), and that  $\epsilon$ -N-acetyl lysine and  $\epsilon$ -N-methyl lysine support growth of rats on lysine-deficient diets, although the corresponding  $\alpha$ -N-substituted derivatives are inactive (60, 46). Certainly some mechanism is needed to explain the degradation of lysine *in vivo*, for which deamination appears to be a preliminary step (see later). It is, however, possible that lysine is oxidized by a different enzyme from the usual amino acid oxidases; such an enzyme has been found in turkey liver, and it rapidly oxidizes L-lysine and other basic amino acids (15).

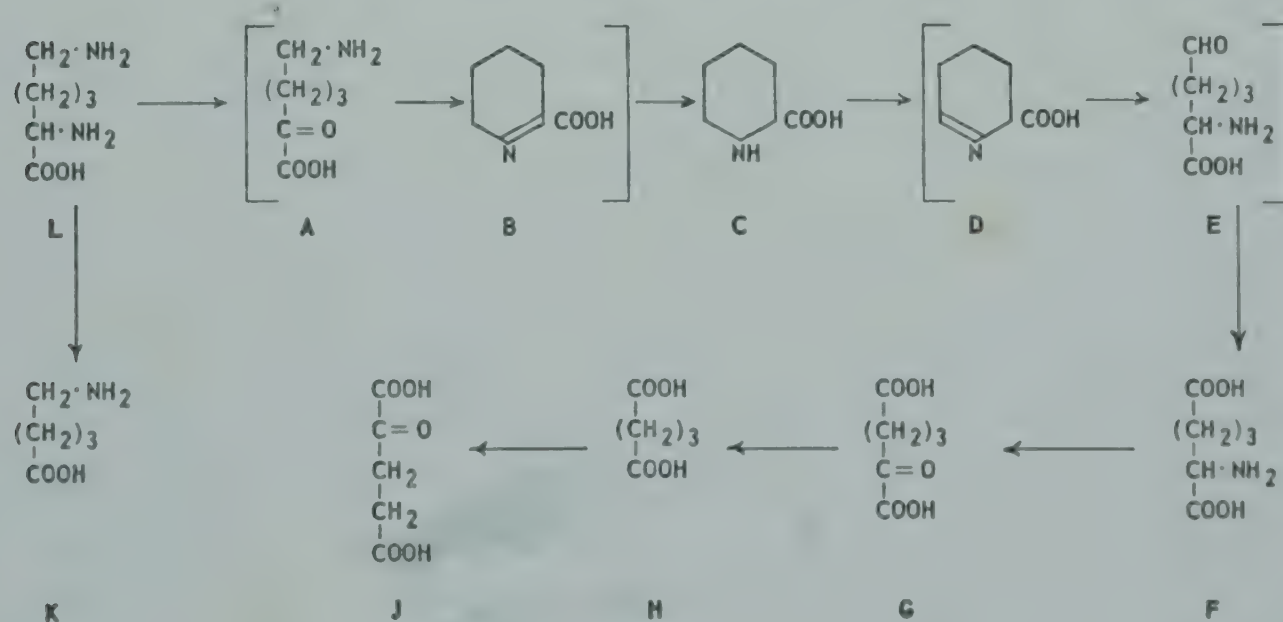
Since in mammals lysine is an essential amino acid, it is evident that mammals lack at least part, if not all, of the synthetic pathways to lysine present in less exacting types of organisms. In considering its degradation, we might recall that lysine differs from the majority of amino acids in that it is neither glucogenic nor ketogenic when fed to animals. Lysine was shown by Borsook and collaborators in 1948 (16, 17) to be converted by liver homogenates to glutaric acid via  $\alpha$ -amino adipic acid and  $\alpha$ -ketoadipic acid. This reaction sequence was confirmed in the whole rat by Rothstein and Miller (72). The technique ("metabolite overloading") used by these authors consisted in feeding the suspected unlabelled catabolite together with radioactive precursor; the catabolite was then isolated from the urine, and the presence of radioactivity was taken to indicate that the administered catabolite had mixed with biologically formed material



and was therefore an intermediate in the reaction sequence under study.

Another intermediate in lysine breakdown has been recently brought to light through the discovery in plants of a new cyclic amino acid, L-pipecolic acid. This was isolated from clover leaves by Morrison (59) and from beans by Zacharius, Thompson, and Steward (102, 47A, 103). Weissmann and Schoenheimer (91) originally suggested in 1941 that this substance might be a deamination product of lysine. This was found to be the case, an efficient conversion of lysine- $\epsilon$ -C<sup>14</sup> to labeled pipecolic acid being demonstrated by Grobbelar and Stewart (47) and by Lowy (51) in plants, and by Rothstein and Miller (70) in the rat. Incorporation of radioactivity into pipecolic acid occurred in the rat from lysine- $\epsilon$ -N<sup>15</sup> but not from lysine- $\alpha$ -N<sup>15</sup> (71, 73), a result demonstrating that it is the  $\alpha$ -amino group of lysine which is removed prior to pipecolic acid formation. The  $\alpha$ -amino nitrogen must then be excreted, since it is not found in any other amino acid. The reaction sequence has been extended further by Rothstein and Miller (Scheme I) by the finding that  $\alpha$ -aminoadipic acid (F) is formed after pipecolic acid (C).

SCHEME I. DEGRADATIVE PATHWAY FOR LYSINE IN THE RAT  
(Rothstein and Miller)



This scheme provides a reasonable mechanism for the conversion of lysine into glutaric acid; so far, direct evidence has not been obtained for the participation of compounds A, B, D, E, and K, the

first four of which are still hypothetical intermediates.  $\delta$ -Amino valeric acid (K) may be formed in small amounts by a side reaction; it may originate from the ketoacid (A), or from lysine itself. Neuberger and Sanger (61) suggested that either  $\delta$ -amino-valeric acid or glutaric acid might be possible intermediates in lysine degradation.

This degradative pathway is evidently not reversible in the rat, as dietary lysine cannot be replaced by either pipecolic acid,  $\alpha$ -amino-adipic acid or  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid (a lysine precursor in *Neurospora*) (85, 42, 64). The two latter compounds act as lysine antagonists, inhibiting growth and hemoglobin formation (64). No isotope interchange occurred between labeled  $\alpha$ -amino-adipic acid and pipecolic acid (71).

The mechanism of oxidative deamination of lysine has not yet been elucidated. If the primary product is  $\alpha$ -keto- $\epsilon$ -aminocaproic acid (A), it would be rapidly converted by ring closure to  $\Delta^1$ -dehydropipecolic acid (B). The keto acid (A) has been prepared and reduced to pipecolic acid by Meister (56), but it has not been tested as an intermediate for rats. In view of its rapid cyclization, it might act as a precursor of pipecolic acid, whether it were a true intermediate or not.

Lysine differs from many amino acids in that the D-isomer and its derivatives do not replace dietary L-lysine for the rat (9, 62). The carbon skeleton of D-lysine is not incorporated into any amino acids of the body proteins (68, 74), but there is some slow loss of  $\alpha$ -amino nitrogen to the tissue proteins (68). This biological inactivity of D-lysine points to a deficiency in available oxidation and transamination mechanisms, by which the other D-amino acids are probably converted to the L-form via the keto acid. The peculiar position of lysine in metabolism may well be due to this deficiency; evidently the  $\alpha$ -keto derivative does not reaminate directly, possibly because of rapid ring closure. Reamination at the  $\alpha$ -carbon atom appears to occur indirectly by amino group transfer from the  $\epsilon$ -carbon through ring closure and subsequent reopening. In the rat, the first identifiable product,  $\alpha$ -aminoadipic acid (F) is not directly available for anabolic reactions, but is degraded via the keto acid (G) to glutaric



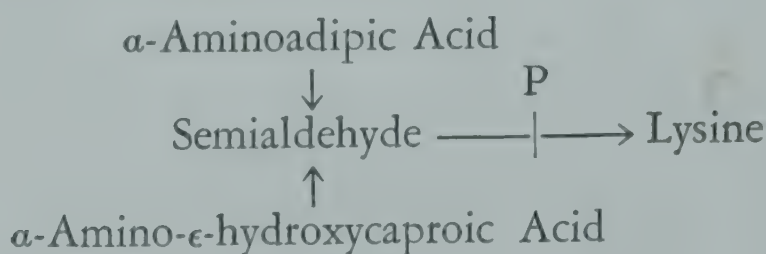
acid (H) and  $\alpha$ -ketoglutaric acid (J). Some transfer of the  $\alpha$ -amino group of  $\alpha$ -aminoadipic acid may take place with citrulline, yielding arginine, since this system has been found to operate in vitro (36).

### MICROORGANISMS

Lysine can be synthesized by a large variety of the less exacting microorganisms, and is an essential nutrient for various exacting bacteria and protozoa. Since relatively little is known about lysine biosynthesis, it is not possible to say whether these exacting organisms are deficient in just one of the enzymes involved in its synthesis [as has been found with some other essential metabolites (50)], or whether a whole chain of reactions is missing. Various artificially induced lysine-requiring mutants have enabled a few steps in the biosynthetic route to be elucidated, while the use of radio isotopes in both exacting and non-exacting organisms is yielding information as to possible precursors and degradation products.

#### Molds

Certain lysine-requiring mutants of *Neurospora crassa* utilized  $\alpha$ -aminoadipic acid instead of lysine (58), while  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid (R, Scheme III) was a less effective growth factor (45). The successive requirements of the various mutants are satisfied by the following Scheme II.

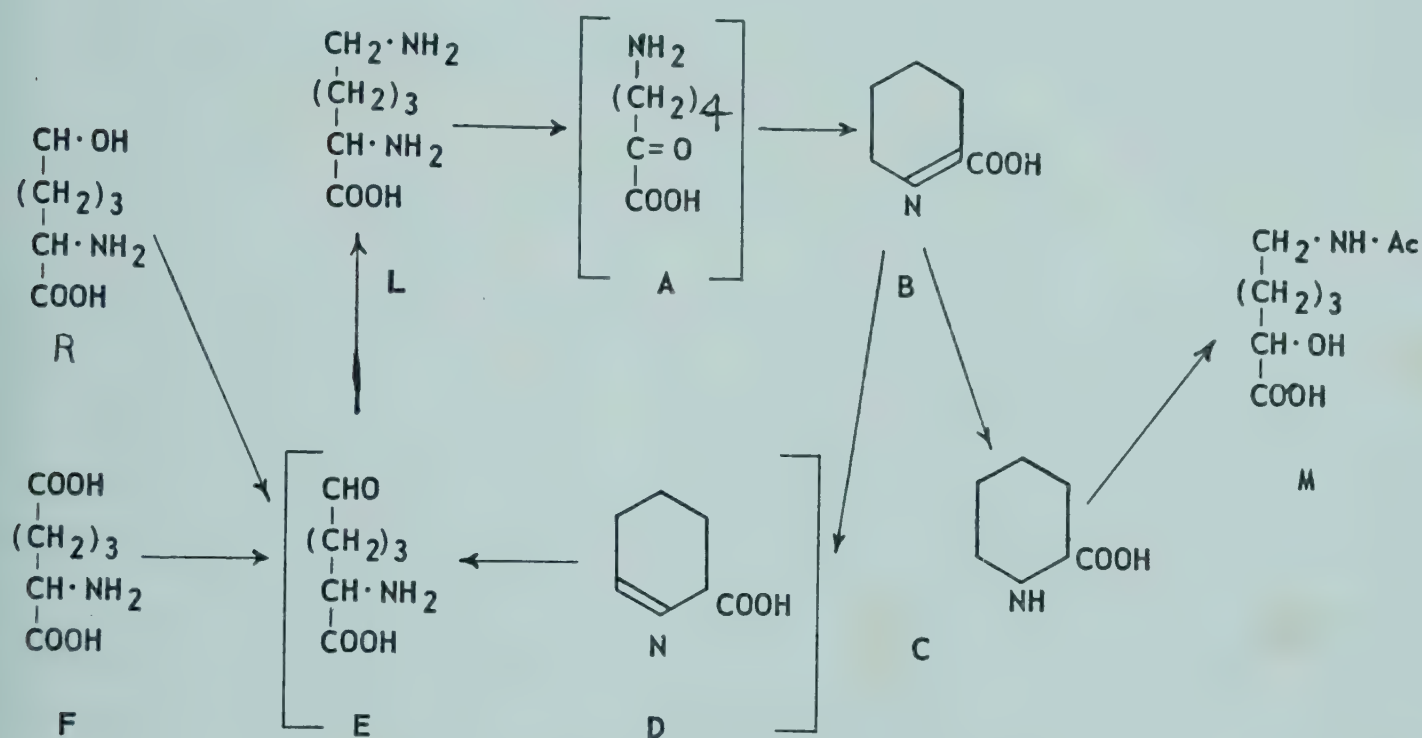


Scheme II

Windsor (92) found that Scheme II represents the only biosynthetic route to lysine, as judged by the complete incorporation of  $\alpha$ -aminoadipic acid- $\epsilon$ -C<sup>14</sup> into lysine. No interconversion to other amino acids was found, indicating that the degradative route to  $\alpha$ -ketoglutaric acid via glutaric acid (Scheme I), found in the rat, does not occur in *Neurospora*.

The use by Schweet and coworkers (77, 78) of the "metabolite overloading" technique with *Neurospora* mutants has shown that here again, as in the rat, pipecolic acid is a catabolite of lysine and cannot substitute for lysine. However, in contrast to the rat, there was in *Neurospora* no conversion of pipecolic acid or lysine to  $\alpha$ -aminoadipic acid. Pipecolic acid was rapidly metabolized by some unidentified reaction which caused it to disappear from the growth medium. The only other main catabolite of lysine isolated from the cells or culture fluid was  $\alpha$ -hydroxy-N-acetyl- $\epsilon$ -aminocaproic acid (M, Scheme III).

SCHEME III. LYSINE METABOLISM IN NEUROSPORA  
(Schweet, Holden, and Lowy)



The L-amino acid oxidase of *Neurospora* is known to oxidize lysine (8), the product being the cyclic  $\Delta^1$ -dehydropipecolic acid (B, Scheme III) (77). Mutants which do not respond to any compound except lysine (i. e. with block at P, Scheme II) are unable to use D-lysine, but those blocked before this point can grow on D-lysine. This suggests that a transamination system for lysine may exist in *Neurospora*. This transamination could be explained by a cycle (Scheme III, proposed by Holden and Schweet et al., 78) of which the first steps to pipecolic acid (C) are similar to those in the rat. The transfer of the  $\epsilon$ -amino group to the  $\alpha$ -position is



effected on opening of the ring—the product being in this case ultimately lysine, and not  $\alpha$ -aminoadipic acid (F) as in the rat. It therefore appears as if the hypothetical intermediate,  $\alpha$ -aminoadipic- $\epsilon$ -semialdehyde (E), or a derivative thereof, might be providing an acceptor for an amino group at the  $\epsilon$ -position, and thus forming lysine. This type of transamination reaction is that suggested by Meister (57).

Mutants of *Neurospora* requiring compounds prior to  $\alpha$ -aminoadipic acid are not available, so that little is known about its precursors. However, a series of lysine-requiring mutants of an *Ophiostoma* (10) show that the reaction sequence of Scheme II applies in this organism. Here,  $\alpha$ -ketoadipic acid replaced  $\alpha$ -aminoadipic acid, although it would not do so for *Neurospora* mutants (92), possibly because the blocks in *Neurospora* were not before  $\alpha$ -ketoadipic acid.<sup>1</sup>

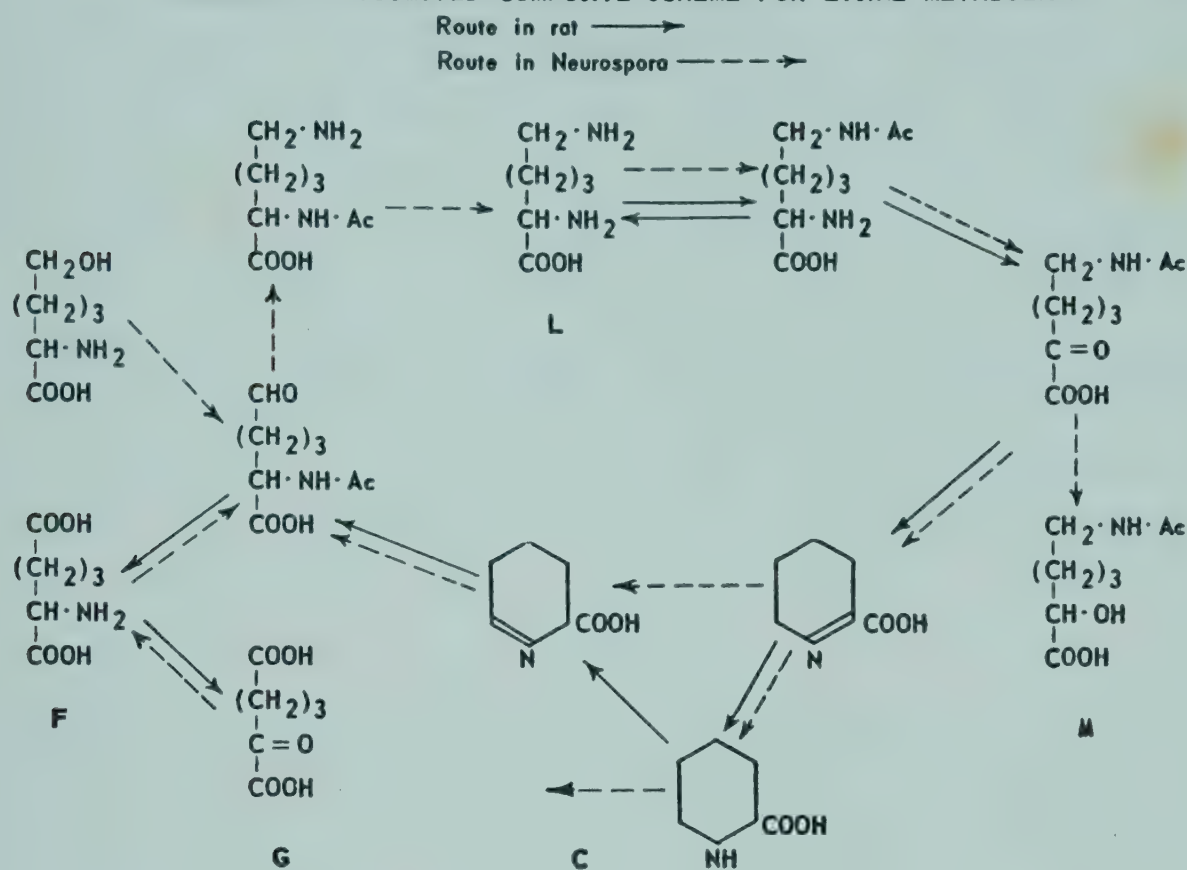
### *Comparison of Neurospora and Rat*

Both pipercolic acid and  $\alpha$ -aminoadipic acid are concerned in the lysine metabolism of *Neurospora* and the rat, and therefore there must be some underlying similarity in the two metabolic pathways. The main differences lie in the behavior of the two organisms towards L-aminoadipic acid and D-lysine, which are precursors of L-lysine for *Neurospora* but not for the rat. I have put together a composite picture (Scheme IV) of all the known facts or theories of metabolism in both organisms. The slight differences in the positions of pipercolic acid (C) are not significant, since the available data do not provide evidence to choose between the two sets of proposed reactions, both of which are equally possible on paper. The main cause of the fundamental differences between *Neurospora* and the rat appears to be the behavior of  $\alpha$ -aminoadipic acid- $\epsilon$ -semialdehyde—which I have stabilized in the scheme by acetylation of the amino group. This compound, or a derivative, is apparently aminated to lysine in *Neurospora* and is oxidized to  $\alpha$ -aminoadipic acid (F) in

<sup>1</sup> Ehrensvärd and co-workers (5A) have published results of a study of labelled acetate incorporation into *Neurospora*, showing that the labelling pattern of lysine resembles that previously obtained for yeast (39) but not for *E. coli* (23).

the rat. Since none of the enzymes involved in these reactions has been isolated or identified, it is not possible to say if the aminating enzymes are actually deficient in the rat, or if the overall enzyme balances are changed in direction by associated coupled reactions.

SCHEME IV. SUGGESTED COMPOSITE SCHEME FOR LYSINE METABOLISM



Acyl groups are inserted on either  $\alpha$ - or  $\epsilon$ -amino groups at the stages of the cycle where a deamination or a reamination would occur. I have tentatively proposed these preliminary acylations for the following reasons: (1) greater susceptibility of the derivatives to deamination and transamination, possibly by prevention of ring closure at the keto groups (see p. 465); (2) occurrence of an  $\epsilon$ -N-acetylated lysine derivative in *Neurospora* (78); (3) analogy with ornithine biosynthesis, from glutamic acid through  $\alpha$ -N-acetylamino (89, 90); (4) replacement of dietary lysine for the rat by  $\epsilon$ -N-acetylated lysine derivatives, but not by  $\alpha$ -N-acetyl compounds (60); (5) lability in the rat of the acetyl group of  $\epsilon$ -N-acetyl-lysine (14A).

### Yeast

The experimental approach to lysine biosynthesis in yeast has of necessity been quite different from that used in *Neurospora*, since lysine is not a growth factor for yeast, and no mutants are available.



The method usually adopted has been to grow yeast on labelled  $\text{CO}_2$ , acetate, or other metabolite, and to examine the distribution of labelling in the lysine isolated from the cells.

Ehrensvar and coworkers (38, 39) showed that lysine, together with leucine and histidine, differed from all other amino acids synthesized by yeast in that its carboxyl group was not derived from respiratory  $\text{CO}_2$ . Ehrensvar, (38, 39), Gilvarg and Bloch (44), Strassman and Weinhouse (86), and Abelson and Vogel (pers. commun.) found that the lysine carboxyl- and  $\epsilon$ -carbons were derived from acetate carboxyl, while the four intermediate carbon atoms originated from the acetate methyl group. Strassman and Weinhouse interpret their results as showing a direct incorporation of an intact acetate skeleton into the carboxyl- and  $\alpha$ -carbon; the origin of the other carbons is uncertain, but was not succinate.

### *Bacteria*

Lysine is an essential metabolite for many strains of the nutritionally exacting bacteria such as streptococci, lactobacilli, and *Leuconostoc*, and is not replaced by either  $\alpha$ -aminoadipic acid or  $\alpha$ -hydroxy- $\epsilon$ -aminocaproic acid (42, 69, 54, 85). This failure to use the known lysine precursors may mean that one or more enzymes of the necessary reaction sequence is missing, or that the substances were impermeable to the bacteria, or that a different route of lysine synthesis exists. The latter possibility was originally suggested for *E. coli* by Ehrensvar and coworkers (23) on the basis of a comparison of the incorporation of labelled acetate into amino acids of yeast and *E. coli*, lysine being one of the few amino acids which failed to show a general similarity of biosynthesis in both organisms. In *E. coli*, lysine carboxyl was probably derived from respiratory  $\text{CO}_2$ , as in the case of the other amino acids, while in yeast and *Neurospora* it was not (see also 3).

### *Diaminopimelic acid as a lysine precursor*

The bacterial amino acid, diaminopimelic acid,  $\text{COOH} \cdot \text{CH}(\text{NH}_2) \cdot (\text{CH}_2)_3 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$ , is being studied as a possible lysine precursor in bacteria. The work on its metabolism which I will

describe was originally carried out by Dr. D. L. Dewey and is now being continued by Dr. D. S. Hoare.

Diaminopimelic acid was originally isolated from *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*, (95) and was also identified in other bacterial products (7, 94 footnote). The *meso* (D-L) configuration was proposed for this amino acid (95, 97, 98). It has since been found to be widely distributed among bacteria (100), for it occurs in all bacteria examined except for the gram-positive cocci and certain *Streptomyces* (Table 1). In some of the

TABLE 1  
DISTRIBUTION OF DIAMINOPIMELIC ACID (DAP) IN MICROORGANISMS

Organism	No. of species examined	Number of strains	
		DAP present	DAP absent
Gram-negative Eubacteriales	8	46	0
Micrococcaceae	3	0	6
Lactobacteriaceae			
Streptococcus	3	0	3
Diplococcus	1	0	1
Leuconostoc	1	0	1
Lactobacillus	2	2	0
Propionibacterium	7	7	0
Corynebacteriaceae	2	2	0
Bacillaceae	7	6	1
Mycobacteria	4	4	0
Mycelial actinomyces	4	10	4
Myxobacteria	2	2	0
Blue-green algae	3	3	0
Other algae	6	0	6
Fungi	19	0	19
Protozoa, yeasts, plant viruses	7	0	7

latter organisms it was replaced by a homologue which may be a methyl-diaminopimelic acid (96). Concentrations of diaminopimelic acid varied from about 0.02% to 2.0% dry wt. of the whole cells. Diaminopimelic acid was confined exclusively to the bacteria and the closely related blue-green algae, being absent from all the specimens examined of yeasts, algae, fungi, or other microorganisms.

The function of diaminopimelic acid in bacterial cells appears to be multiple, judging by its wide distribution in various cellular frac-



tions. It occurred in the soluble amino acid fraction of some, but not all bacteria, but was present mainly in the insoluble residues (94, 100, 14, 65). In *C. diphtheriae* and *E. coli*, high concentrations of diaminopimelic acid occurred in the insoluble cell wall fraction (49, 22, 76), but the soluble cytoplasmic contents of *E. coli* also contained the amino acid (100), even after removal of various nucleoprotein fractions by high speed centrifugation (these nucleoproteins contained no diaminopimelic acid; Work, unpub.). Soluble protein fractions from *C. diphtheriae* and *M. tuberculosis* contained diaminopimelic acid (100, 43), as did an extracellular peptide from *B. subtilis* spores (67) and also antigenic lipopolysaccharides from *M. tuberculosis* (6, 7).

The first suggestion of a metabolic role for diaminopimelic acid, apart from its function as a stable cell constituent, came from Davis (25) in his search for mutants of *E. coli* (an organism containing diaminopimelic acid). A mutant (173-25) was found which had an absolute requirement for diaminopimelic acid and a relative requirement for lysine. The mutant grew rapidly on a mixture of the two amino acids, slowly on diaminopimelic acid alone, and not at all on lysine alone. Secondary mutants arising from this strain grew slowly on either amino acid, but rapidly on both. Certain mutants having an absolute requirement for lysine (81-83, 26-26, and 81-47) could not utilize diaminopimelic acid as a growth factor (Table 4); but other lysine-requiring mutants with incomplete blocks (81-29, 81-43) grew slowly on a mineral medium, but rapidly with either diaminopimelic acid or lysine.

At the time Davis was doing this work, we had already found in *E. coli* an enzyme decarboxylating diaminopimelic acid (32). This decarboxylase differs from most of the other known bacterial amino acid decarboxylases (40) in that it does not function at acid reactions, having a pH optimum near neutrality (Fig. 1). The decarboxylation product is lysine, which is formed with evolution of 1 mole of  $\text{CO}_2$  per mole of diaminopimelic acid.

The activity of diaminopimelic decarboxylase in *E. coli* and *A. aerogenes* is independent of pH, glucose concentration, or degree of aeration of the medium during growth, while the presence of

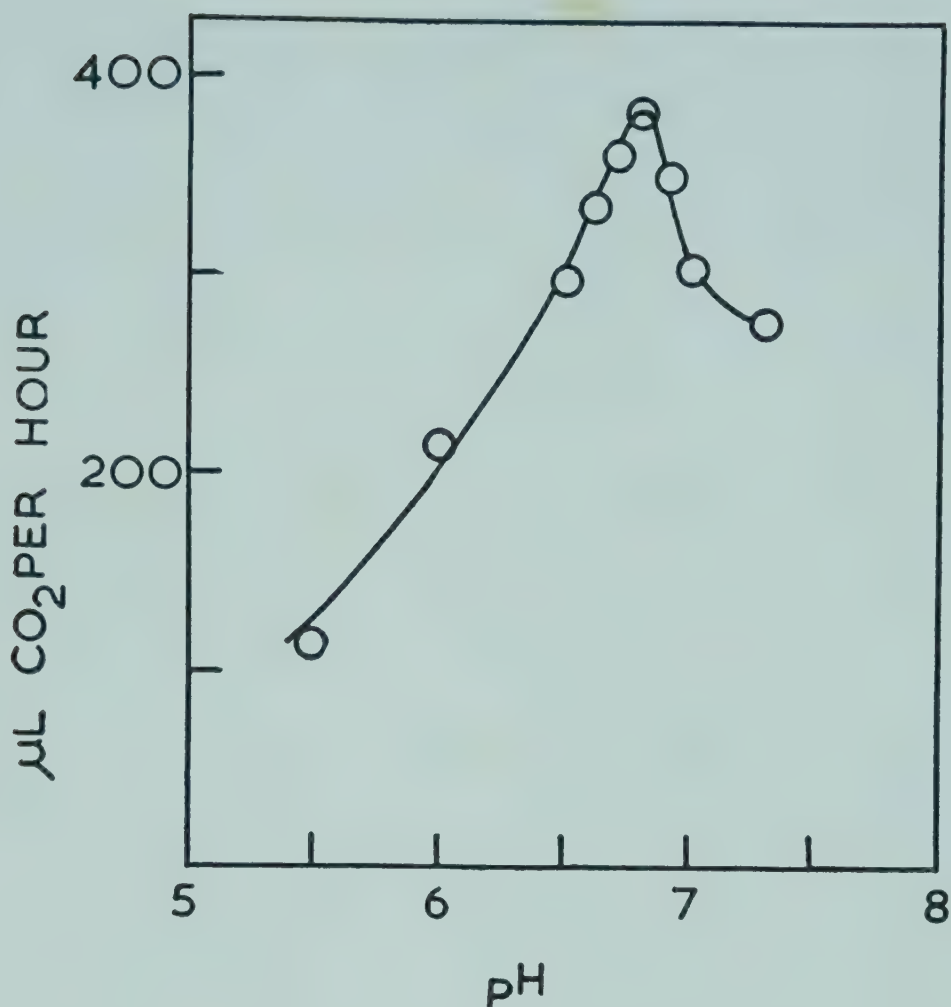
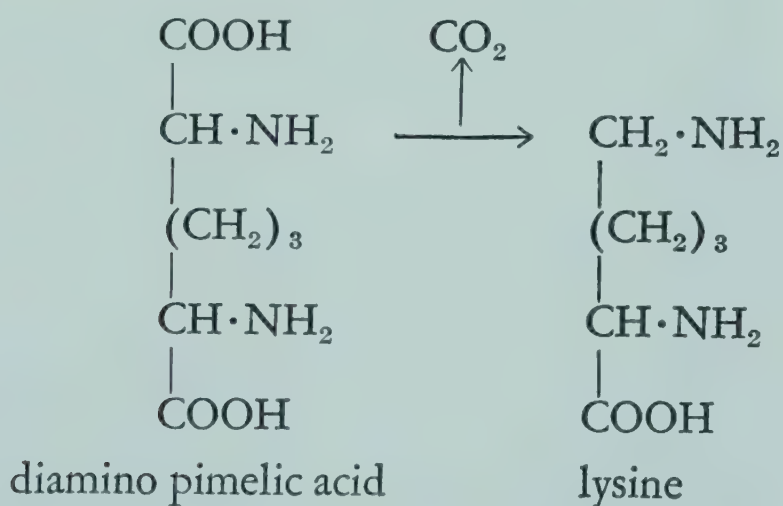


FIG. 1. Effect of pH on the activity of diaminopimelic acid in purified extracts of *A. aerogenes*. (From Dewey, Hoare, and Work, 1934.)



diaminopimelic acid in the growth medium does not increase activity (32, 33, 34). Therefore, diaminopimelic decarboxylase is not an inductive enzyme acting as a protection against an acid environment, as are the other bacterial amino acid decarboxylases which produce basic amines of no known major metabolic function. It appeared to us that diaminopimelic decarboxylase might be a constitutive enzyme, since it was unaffected by external conditions, functioned



at physiological pH, and produced an important metabolite. We therefore asked Dr. Davis if we could examine his lysine-requiring mutants for decarboxylase activity (Table 2), and found that the 3 mutants with absolute lysine requirements had no diaminopimelic decarboxylase, while the mutant requiring diaminopimelic acid had the same enzyme activity as the parent strain (32).

TABLE 2

DIAMINOPIMELIC DECARBOXYLASE ACTIVITIES OF MUTANT STRAINS OF *E. coli*

Strain	Growth requirement	*Q <sub>CO<sub>2</sub></sub> at pH 7.0	
		Diaminopimelic acid	Lysine
ATCC-9637	Simple, parent strain	3.1	16
81-83	Lysine	0	10
26-26	Lysine	0	16
81-47	Lysine	0	12
173-25	DAP (+ lysine)	3.5	20

\* Q<sub>CO<sub>2</sub></sub> =  $\mu$ l. CO<sub>2</sub>/mg. acetone-dried cells/hr. at 37° C.

This finding suggested that the block in the lysine-requiring mutants was at the diaminopimelic decarboxylase stage; this was confirmed by Davis, who showed that all these three lysine-requiring mutants accumulated large amounts of diaminopimelic acid in their growth medium. [This accumulation was later utilized in the preparation of diaminopimelic acid, the culture fluid from one of the mutants providing a source of amino acid (99, 101)].

It has been necessary to prove that the absence of diaminopimelic decarboxylase activity from the mutants was due to a loss of enzyme, and was not the result of permeability changes or inhibition. The facts that cell-free extracts of the mutant cells (26-26) show no enzyme activity and do not inhibit other active cell-free extracts from the parent strain, provide the necessary criteria stipulated by Davis to establish that the phenomenon is a true enzyme loss.

Sufficient evidence is now available to establish that, in *E. coli*, diaminopimelic acid is a precursor of lysine. The relevant facts are: (1) isolation of the enzyme carrying out the reaction in one step; (2) finding of mutants in which the enzyme is missing; and (3)

accumulation of the substrate for that enzyme in the culture medium. The alternative explanation, that diaminopimelic acid may be a side-product, enzymatically interconvertible with the true precursor (4), may in this case be discounted, owing to the obligatory one-step nature of the decarboxylation reaction whereby diaminopimelic acid is converted to lysine.

Further investigations of these lysine-requiring mutant cells (26-26) has revealed large amounts of free diaminopimelic acid in the extractable amino acids, whereas in the normal wild-type strain no soluble diaminopimelic acid was found. This building-up of free diaminopimelic acid is to be expected, in view of the large-scale excretion which occurs. The lysine-requiring mutant is notoriously unstable in the absence of lysine from the growth medium, and Hoare (unpub.) has found some correlation between the growth time in a synthetic medium containing a trace of lysine, the accumulation rate of diaminopimelic acid in the medium, and the cellular levels of diaminopimelic decarboxylase in a culture which was reverting after using up the lysine (Table 3). Evidently, after 24

TABLE 3

EFFECT OF GROWTH TIME ON LYSINE-REQUIRING *E. coli* MUTANT (26-26)  
GROWN ON MINIMAL AMOUNTS OF LYSINE (10 MG./L.)

Growth time (hours)	Yield of dry cells (mg./l. of culture fluid)	DAP content of culture filtrate (mg./l.)	DAP decarb. content of cells Q <sub>CO<sub>2</sub></sub>	Free DAP in cells
12	120	24 *	0	+
18	110	45 *	0	
24	120	93 *	trace	
48	200-250⊙	100⊙	4.0	0

\* Estimated by decarboxylation of electro dialysed deproteinized culture filtrate.

⊙ Mean of 4 preparations in which DAP was isolated from 8 l. of culture filtrate (99).

hours, the parent strain containing decarboxylase had begun to grow, growth of mutant with no decarboxylase having ceased at about 12 hours. The amino acid in the medium accumulated most rapidly after growth of the mutant had ceased, but showed little change



after reversion when the parent strain was growing rapidly. This behavior of the enzyme content of the overall culture is in sharp contrast to that of the wild-type strains of *E. coli* (Fig. 2), where there is a sharp peak of enzyme activity at the period of maximum growth (12½ hours at 37° C.), and a very rapid fall thereafter (32, 34).

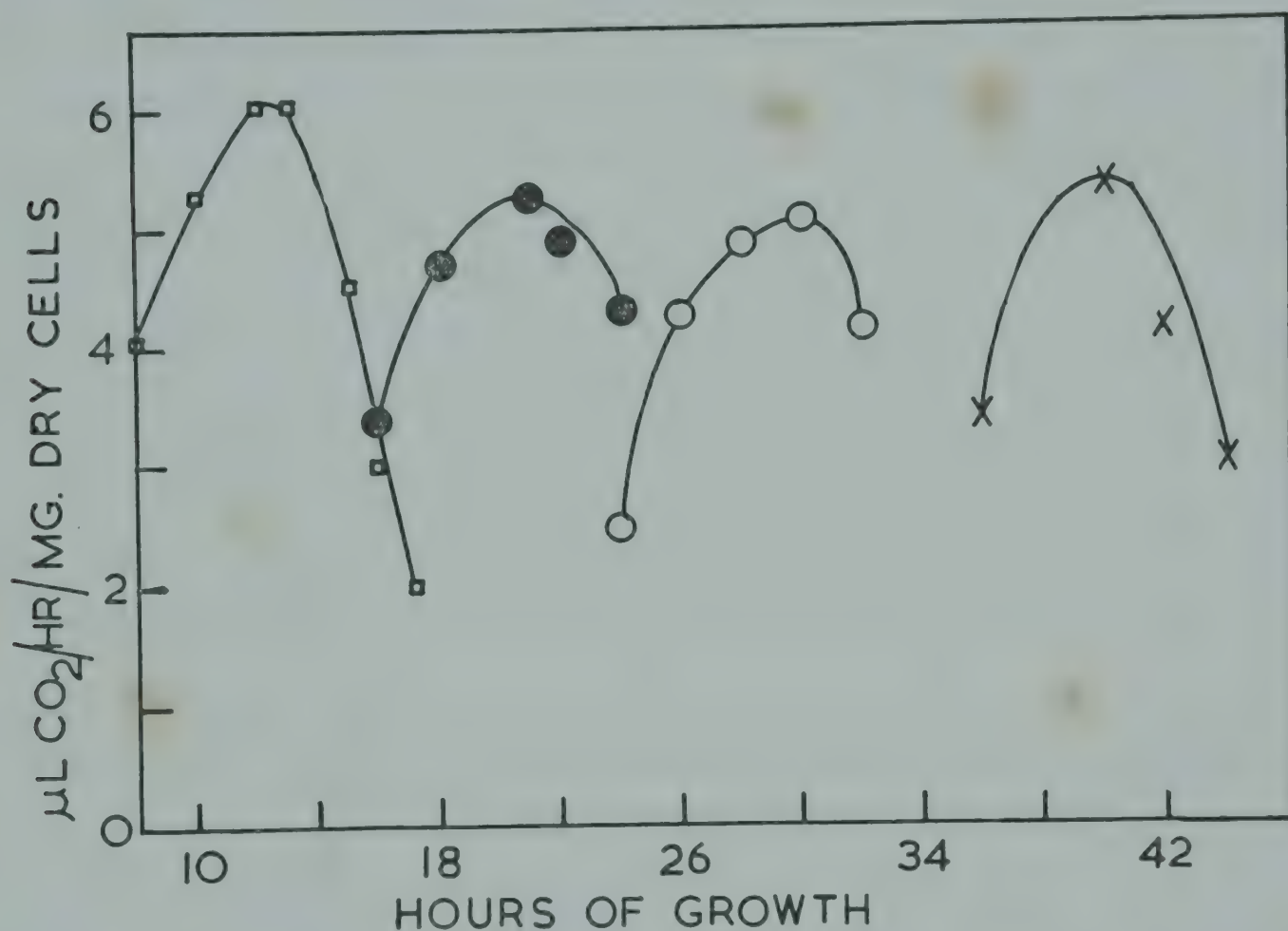


FIG. 2. Effect of growth time and growth temperature on DAP decarboxylase activity in *E. coli*. Cells grown at 37° C., □—□, on nutrient broth and tested at pH 7.2. Cells grown at 18° C., ×—×; at 25° C., ○—○, and at 30° C. ●—● on a salt glucose medium and tested at pH 6.8. (From Dewey, Hoare, and Work, 1934.)

The final decarboxylase figure ( $Q_{CO_2} = 4.0$ ) attained by the 48-hour culture is remarkably high, when it is considered that the reverted cells must have been diluted by almost 50 per cent of their weight by mutants containing no diaminopimelic decarboxylase. The normal  $Q_{CO_2}$  obtained with the wild-type parent culture is 3.1 (see Table 2). The enzymic behavior of this culture on reversion is different from that found by Mitsuhashi (described by Davis, 29) in his study of a mutant lacking the enzyme producing dehydroshikimic acid from dehydroquinic acid. On complete reversion to a

strain growing as fast as the wild type on minimal medium, the cells only contained 20 per cent as much enzyme as the parent strain and continued to accumulate dehydroquinic acid.

*Aspartic acid and threonine as lysine precursors.*

A. Evidence from Mutants of *E. coli*.

Further examination by Davis of his lysine-requiring mutants (Table 4) suggested that both threonine and aspartic acid might

TABLE 4

MUTANTS OF *E. coli* CONCERNED WITH LYSINE AND DIAMINOPIMELIC ACID (DAP)  
(DAVIS, 25)

No. of strain	Amino acid requirement	Amino acid accumulated
173-25 ↓	(1)DAP (absolute) + lysine (relative)	Threonine
173-25c3	(2)Lysine or DAP	Threonine
81-83 } 81-47 } 26-26 }	Lysine	DAP
81-29	(3)Lysine or DAP	—
81-43	(3)Lysine or DAP	Threonine
122-64	Aspartic acid + lysine or DAP	—
39A-21	Threonine, lysine or DAP	—
43-6	Threonine	—

(1) Inhibited by excess lysine.

(2) Accelerated by CO<sub>2</sub> in presence of lysine.

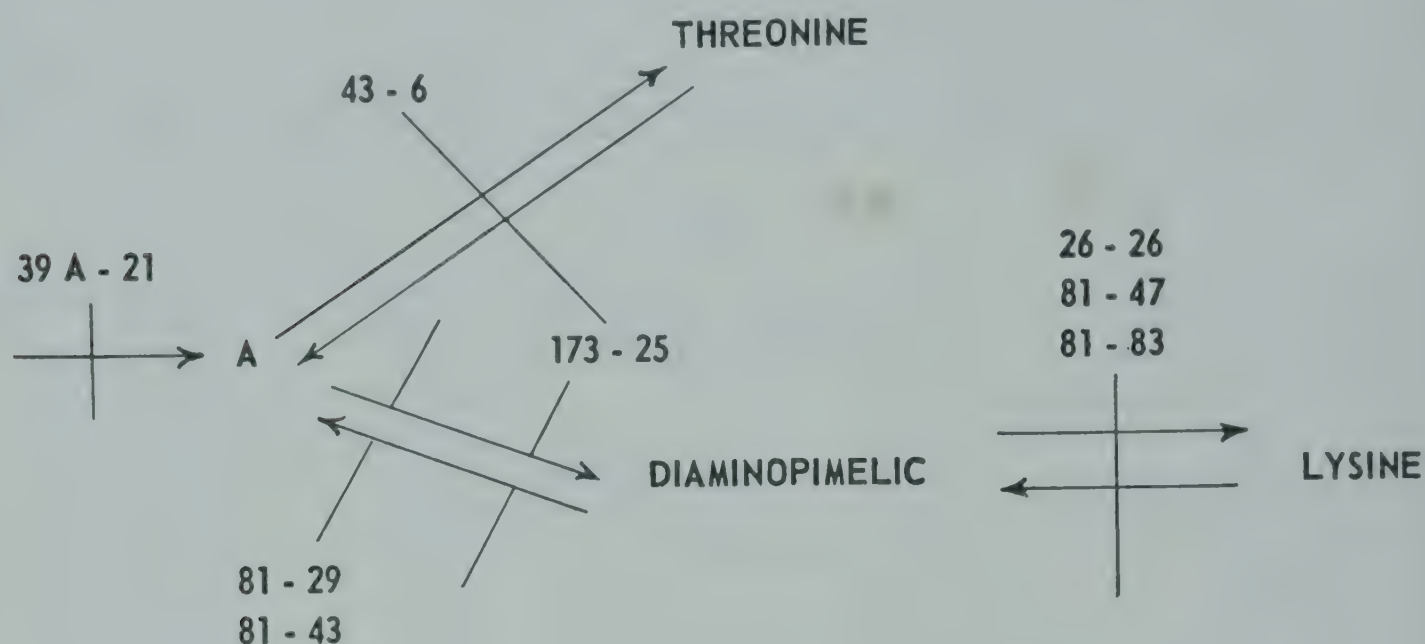
(3) Partial requirement made absolute by aspartic acid.

play some part in the biosynthesis of diaminopimelic acid and lysine (25). Three strains (173-25, 173-25c3, and 81-43) requiring diaminopimelic acid or lysine accumulated threonine in their culture fluid, while threonine was an alternative growth factor to lysine or diaminopimelic acid for 39A-21. Strain 122-64 required L-aspartic acid in addition to diaminopimelic acid or lysine; the partial requirements of strains 81-29 and 81-43 for diaminopimelic acid or lysine were converted to absolute requirements by the presence of aspartic acid—in other words, aspartic acid inhibited in these strains the slow



growth which occurred in the absence of amino acids. None of the lysine-requiring mutants responded to  $\alpha$ -amino adipic acid or  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid.

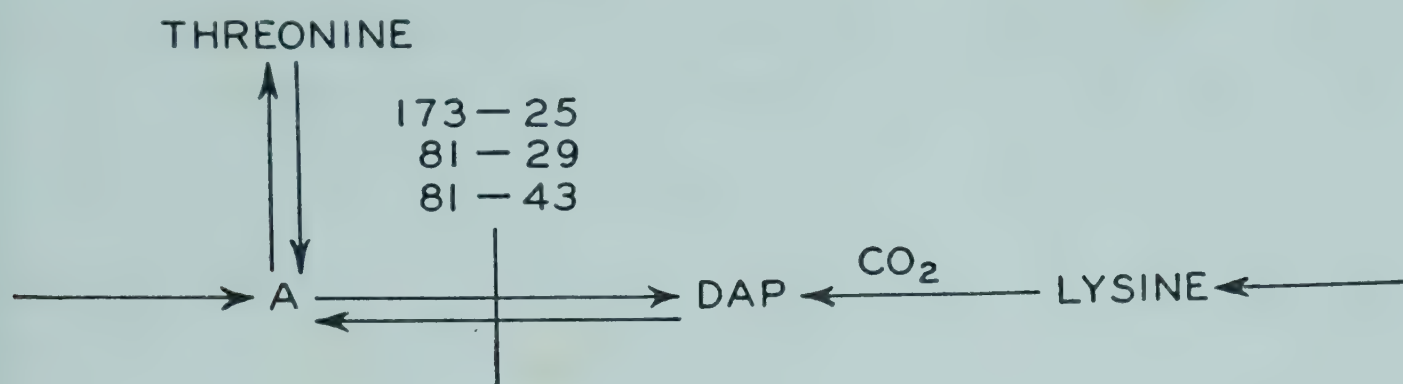
Scheme V, for lysine biosynthesis in *E. coli*, was put forward by Davis to account for his findings.



Scheme V. Lysine biosynthesis in *E. coli* mutants (Davis, 25).

The scheme provides a general explanation for the intervention of threonine in lysine biosynthesis, by postulating a common precursor for threonine and diaminopimelic acid. It does not, however, explain the fact that strains 81-29 and 81-43 will grow slowly in the absence of lysine or diaminopimelic acid; or the behavior of strain 173-25, which requires very little diaminopimelic acid for growth ( $5 \mu\text{g./ml.}$  of medium), but which only attains full growth in the presence of lysine ( $20 \mu\text{g./ml.}$ ). Davis attributes the relative lysine requirement of 173-25 either to a secondary effect, due to partial inhibition by a metabolite accumulated before the metabolic block, or to poor absorption of diaminopimelic acid by the growing cells. Another explanation could be that in *E. coli* an alternative route to lysine exists, and normally supplements the route through diaminopimelic acid which is unable to satisfy all the lysine requirements of the cell because of the low activity of diaminopimelic decarboxylase. When the metabolic block is before diaminopimelic acid, some growth might take place through the use of the alternative

route, the reversal of the decarboxylation reaction producing diaminopimelic acid:



Evidence for this reversal was provided by various secondary mutants of 173-25, where growth in the presence of lysine alone was accelerated by CO<sub>2</sub>.

This partial requirement of strain 173-25 for lysine has its metabolic counterpart in an isoleucine-requiring mutant of *E. coli* (43-37) which had a relative requirement for valine. Rudman and Meister (75 and p. 17) showed that the mutant had lost the main transaminase involved in aminating the keto acids of isoleucine and valine, but that an alternative weaker transaminase for valine (but not isoleucine) still produced valine in sufficient amount to maintain slow growth in the presence of isoleucine alone.

If this alternative route to lysine exists, how can one explain the absolute lysine requirement of the strains lacking in diaminopimelic decarboxylase (81-83, 26-26, and 81-47). The only possible explanation is that the accumulation of diaminopimelic acid inhibits some reaction on the alternative route. The inhibitory effect of added aspartic acid on the growth of 81-29 and 81-43 in the absence of either lysine or diaminopimelic acid could also be due to the blocking of the alternative route, and it may be that the two carboxyl groups of diaminopimelic acid and of aspartic acid are inhibiting the same reaction.

Davis' work in the field of aromatic amino acid synthesis has produced a clue to a cofactor for lysine biosynthesis. A series of mutants was found requiring various aromatic factors all derived from the common precursor shikimic acid. *p*-Hydroxybenzoic acid was one of these compounds and for it the organisms showed a relative



requirement in trace amounts (24). *p*-Hydroxybenzoic acid could be replaced by methionine or homoserine and lysine or diaminopimelic acid (27, 28). The relative requirement was converted to an absolute one by traces of aspartic acid in the medium (26).

This relationship of methionine to lysine may be understood if one recalls that both threonine and methionine have homoserine as a precursor. It is further exemplified by various mutants found in other laboratories which require both methionine and lysine.

#### B. Evidence from Isotope Incorporation in *E. coli*.

An extensive study of amino acid interrelationships in *E. coli* has been made by Abelson and coworkers (1, 2, 3) by employing "isotopic competition." This technique utilizes the fact that certain unlabelled amino acids presented to *E. coli* growing in the presence of  $C^{14}$ -labelled glucose or  $CO_2$  depressed the incorporation of  $C^{14}$  into the cellular proteins. Thus, depression of radioactivity in a certain amino acid (usually observed on a paper chromatogram) indicated that the exogenous unlabelled material was acting as a precursor for that amino acid in preference to any products of glucose catabolism. By this technique, aspartic acid was found to produce a "family" of amino acids, which included threonine, methionine, isoleucine, diaminopimelic acid, and lysine; whereas glutamic acid was incorporated into proline and arginine. In the case of threonine, interconversion was complete and was via homoserine, since exogenous homoserine, as well as aspartic acid, depressed the radioactivity of threonine. The figures for isoleucine, diaminopimelic acid, and lysine did not indicate that aspartic acid was their only precursor, and could be due to the existence of alternate biosynthetic routes to these amino acids. Since added diaminopimelic acid caused no depression of lysine radioactivity, the suggestion was made that diaminopimelic acid is not on the direct biosynthetic route to lysine. However, this comment is without justification, since added diaminopimelic acid had no effect on the radioactivity of cellular diaminopimelic acid, and also since the radioactivities of diaminopimelic acid were very low, due to the small amounts of this amino acid in *E. coli*.



This negative result in regard to diaminopimelic acid incorporation cannot have been due to lack of penetration into the cell, since Cowie (pers. commun.) has found that the resting cells were as freely permeable to diaminopimelic acid as they were to other amino acids or to inorganic ions (21). It appears that the question of the availability of a substrate to bacteria cannot always be solved by measuring uptake by cells: a second factor may sometimes be involved which can present a barrier to the free access of substrates to the enzyme sites. The strain (B) of *E. coli* used in the isotopic competition experiments evidently presented an unusually high barrier to access of lysine and diaminopimelic acid to their decarboxylases, as compared with other strains. Hoare observed that the effect of acetone-drying of strain B on lysine decarboxylase activity ( $Q_{\text{CO}_2}^{\text{lysine}}$ , fresh = 31, dried = 300) was much greater than in a strain grown by Gale and Epps (41) under the same growth conditions ( $Q_{\text{CO}_2}^{\text{lysine}}$ , fresh = 254, dried = 430). No diaminopimelic decarboxylase activity was detected in fresh *E. coli* B; after acetone-drying the level was  $Q_{\text{CO}_2}^{\text{DAP}} = 9.0$ .

### C. Growth Requirements and Isotope Incorporation in Other Organisms

So far, the only bacterial species discussed in this review has been *E. coli*, an organism which can synthesize its own lysine. In the more exacting lactobacilli (which contain diaminopimelic acid), many of which show relative or absolute requirements for lysine, a relationship between aspartic acid, threonine, and lysine also exists. In *L. arabinosus*, aspartic acid could be replaced for growth in a defined medium by a mixture of threonine, lysine, bicarbonate, and biotin (69). In the absence of any one of these substances, aspartic acid became a growth factor, but could be "spared" by each of the other substances. *L. casei* required lysine under all circumstances, but threonine, biotin, and bicarbonate could replace or spare aspartic acid. Either lysine or threonine antagonized the inhibitory effect of cysteic acid on aspartic acid utilization by both organisms.

In certain other lactobacilli, alanine, threonine and lysine could be replaced by pyridoxine, either alone or with excess  $\text{CO}_2$  (52, 81).



In streptococci, organisms lacking diaminopimelic acid, aspartic acid appears to be related biosynthetically to lysine, but there is no evidence that threonine is connected. Pyroxidine can, under certain conditions, replace lysine or aspartic acid for growth of *Streptococcus faecalis* R (52). In another strain (6057), lysine was replaceable by a mixture of pyridoxine, aspartic acid, asparagine, proline, tyrosine, inositol, and choline (53). An incorporation of aspartic acid into protein lysine was demonstrated (53) in cells grown without lysine in the presence of  $C^{14}$ -aspartate, 27 per cent of the total protein radioactivity being found in lysine.

*Summary of knowledge of lysine biosynthesis in E. coli*

In order to integrate the facts already presented on lysine biosynthesis in bacteria, it is necessary first to consider the known facts on the synthesis of threonine from aspartic acid. This has been worked out in yeast extracts by Black and colleagues (11, 12, 13), who found the following reaction sequence: aspartic acid  $\rightarrow$   $\beta$ -aspartyl phosphate  $\rightarrow$  aspartic- $\beta$ -semialdehyde  $\rightarrow$  homoserine (see p. 58). Cohen and collaborators (20, 48, 63) have found that resting-cell suspensions of *E. coli* can carry out the direct conversion of aspartic acid to threonine via the following steps: aspartic acid  $\rightarrow$   $\beta$ -aspartyl phosphate  $\rightarrow$  homoserine  $\rightarrow$  threonine.\*

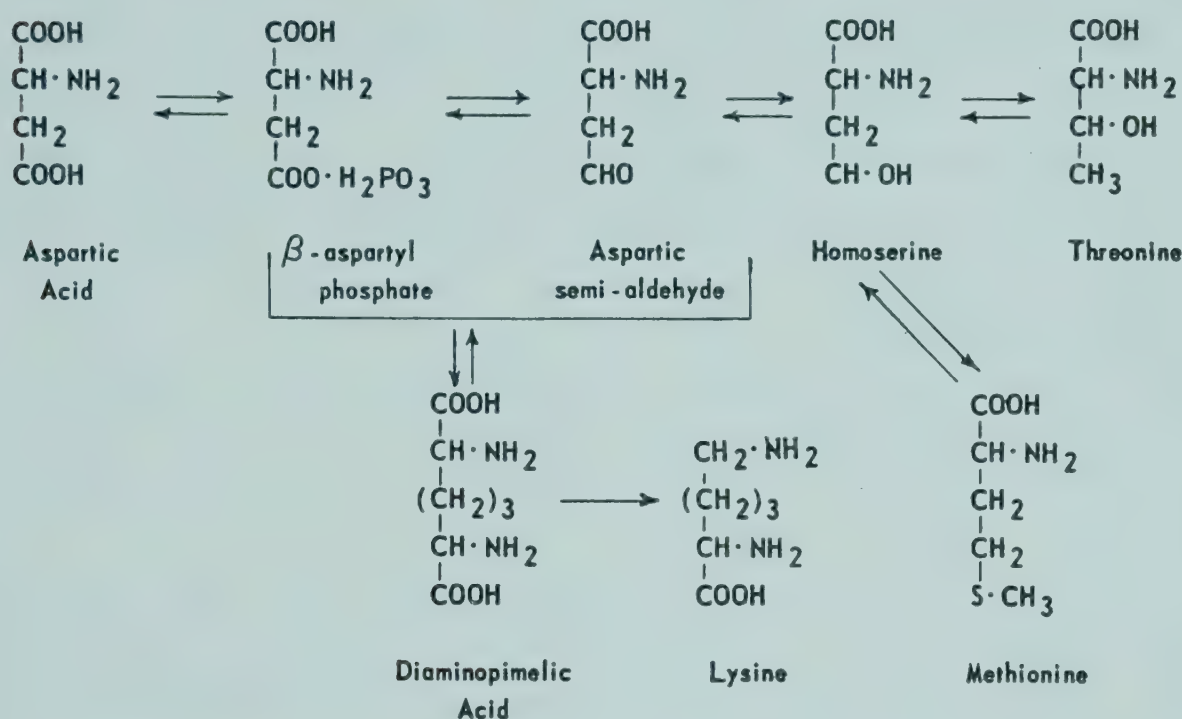
These experiments show that the route to homoserine (and threonine) in *E. coli* resembles that in yeast. The overall pathway from labelled  $CO_2$  or acetate for threonine biosynthesis is generally considered to be the same for *E. coli*, yeast, *Neurospora* and *B. subtilis* (23, 39, 2, 3, 87, 88, 30, 5A). This involves primary  $CO_2$  fixation into both carboxyls of aspartic acid, followed by conversion to threonine labelled in the same position as aspartic acid.

In *E. coli* but not in yeast, lysine follows the same pathway as threonine (23, 39, 3). Fixed  $CO_2$  is identified in the carboxyl group which is derived from the  $\alpha$ -carboxyl of aspartic acid (not from the acetate carboxyl as in yeast). Scheme VI presents an attempt at integration of the known facts.

\* In his contribution to this symposium (p. 58), Black suggests that Cohen has not shown that  $\beta$ -aspartyl phosphate is an intermediate for *E. coli*.

There is as yet no direct evidence for the participation of these aspartyl derivatives in diaminopimelic biosynthesis. We know, however, that lysine has a common precursor with threonine; homoserine was shown by Abelson (1) not to be a lysine precursor, but is known to form methionine. Since both methionine and

SCHEME VI. POSSIBLE ROUTE FOR LYSINE BIOSYNTHESIS IN *E. COLI*.



diaminopimelic acid have a common cofactor requirement (27, 28), there must be one intermediate common to lysine, threonine, and methionine biosynthesis. This might be either  $\beta$ -aspartyl phosphate, aspartic semialdehyde, or some other active aspartic acid derivative. It is interesting to note that in this review  $\omega$ -semialdehydes of three dicarboxylic amino acids have been mentioned as intermediates in amino acid biosynthesis.

The metabolic inertness of exogenous lysine in *E. coli* was shown by an investigation of the incorporation of  $\text{C}^{14}$ -carboxyl-lysine by *E. coli* grown on lactate and lysine (79). No isotope was found in any fraction other than in the lysine of bacterial protein and non-protein fractions: even respiratory  $\text{CO}_2$  was non-radioactive. Unfortunately diaminopimelic acid was not examined, so it is not known whether any interconversion with lysine occurred. At low levels of lysine in the medium, about 55 per cent of the bacterial



lysine was derived from exogenous lysine, but after ten-fold increase of the lysine content of the medium, still only 77 per cent of the cellular lysine was radioactive. Evidently, endogenous production of lysine was not entirely eliminated by high levels of lysine in the growth medium. This supports the proposed alternative route of lysine biosynthesis; one route may be dispensed with if the cell is presented with lysine in the growth medium, and the other may be utilized continuously even in the presence of added lysine.

The nature of this alternative route is completely unknown, but since lysine is actively transaminated by *E. coli* (39A), one might speculate that transamination could be one of the steps on the route.

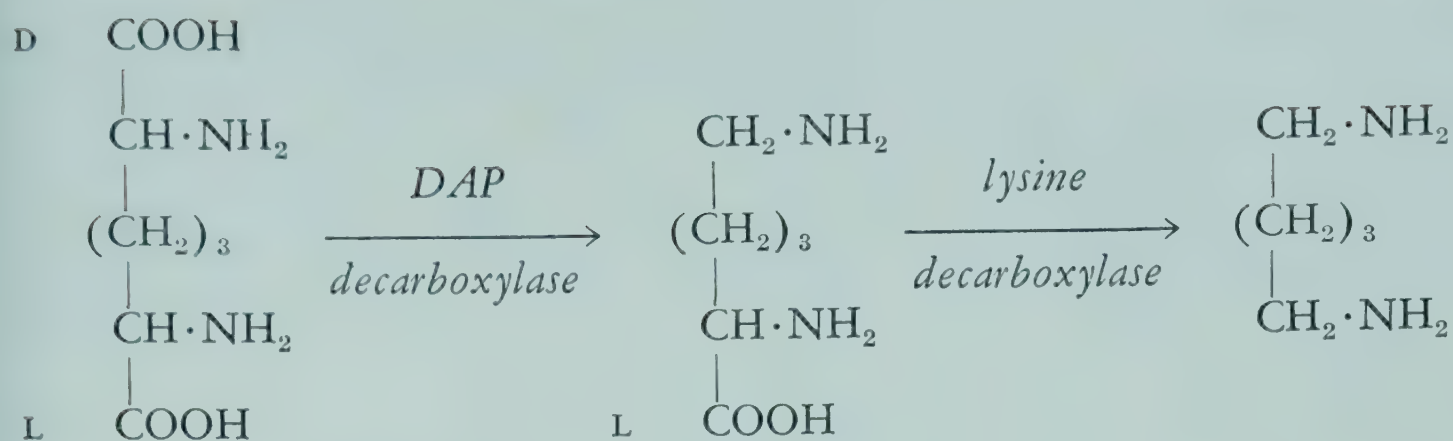
### *Lysine degradation.*

Very little is known about the pathway of lysine degradation in microorganisms, which can often utilize lysine as a source of nitrogen and energy. Recently Stadtman (82, 83, 84) has found in *Clostridium* an interesting set of reactions (which she will describe hereafter), whereby lysine is decomposed anaerobically to acetate, butyrate, and ammonia. A similar type of reaction has been described in mixed cultures of *E. coli* (35).

### *Further properties of diaminopimelic acid decarboxylase.*

I propose next to describe some more of our work on diaminopimelic decarboxylase, in so far as it is related to lysine biosynthesis. Only part of this work has been published (33, 34).

Cell-free extracts of the enzyme from *Aerobacter aerogenes* were, after fractionation with acetone, free from other amino acid decarboxylases. Such preparations decarboxylated meso-(D-L)-diaminopimelic acid to lysine. The unfractionated enzyme system in acetone-dried cell suspensions converted meso-diaminopimelic acid to cadaverine because of the additional presence of lysine decarboxylase. This proves that the lysine formed from meso-diaminopimelic acid was the L- and not the D-form, since lysine decarboxylase does not attack D-lysine. It follows, then, that the point of attack by diaminopimelic decarboxylase is the carboxyl group in the D-configuration.



Decarboxylation of *meso*-diaminopimelic acid.

Diaminopimelic decarboxylase was partially activated by pyridoxal phosphate, and the inhibition by cyanide was reversed by dialysis, facts suggesting that pyridoxal phosphate is a coenzyme. No dissociation of coenzyme was achieved by dialysis, while salt fractionation produced irreversible destruction. The enzyme has a sensitive SH group and is inactivated in aqueous solutions in the absence of other thiols.

The specificity in diaminopimelic decarboxylase is very marked. So far no amino acid other than naturally occurring diaminopimelic acid has been found to be attacked. All three of the lower homologues of diaminopimelic acid and five of its higher homologues (80) were tested; none of them was attacked by diaminopimelic decarboxylase, nor did any of them inhibit the enzyme. Naturally occurring analogues such as  $\alpha,\epsilon$ -diamino- $\beta$ -hydroxypimelic acid (93), methyldiaminopimelic acid (96, 97) *meso*-lanthionine (5) and dipicolinic acid (66) were also not decarboxylated; but dipicolinic acid, which has the carbon skeleton of diaminopimelic acid, produced 45 per cent inhibition when present in equimolar concentration to the substrate.

#### *Diaminopimelic decarboxylase in pyridoxine-deficient E. coli.*

The constitutive nature of diaminopimelic decarboxylase has been deduced from our inability to alter its levels in the cell by changes in growth conditions. No knowledge of its dispensability to the cell is available, other than the fact that, when it is absent from certain *E. coli* mutants, growth ceases. It was therefore of interest to investigate the effect of pyridoxine deficiency on diaminopimelic decarboxy-



lase activity This was carried out by growing a pyridoxine-requiring mutant of *E. coli*, either in the minimal concentration of pyridoxine necessary for growth, or in a mixture of 17 amino acids (including lysine) which could replace pyridoxine. Table 5 shows that substantial reductions in the pyridoxine content of the cells (kindly

TABLE 5

EFFECT OF PYRIDOXIN DEFICIENCY ON DECARBOXYLASE CONTENTS OF A PYRIDOXIN-REQUIRING MUTANT OF *E. coli*.

Py = pyridoxine. PyalPO<sub>4</sub> = pyridoxal phosphate

Supplement to minimal medium	Cellular Py. content ( $\mu$ g. Py. HCl /g. dry wt.)	Decarboxylase activity (Q <sub>CO<sub>2</sub></sub> )			
		Diaminopimelic		Lysine	
		Alone	+ PyalPO <sub>4</sub>	Alone	+ PyalPO <sub>4</sub>
I. Py (20 $\mu$ g./l.)	10.3	6.1	10.1	0	0
II. Py (20 $\mu$ g./l.) + lysine	16.5	2.2	5.7	9.8	28.8
III. Amino acid mixture + lysine	21.0	4.9	4.9	17.2	17.5
IV. Py (500 $\mu$ g./l.)	91.6	5.0	5.6	0	0
V. Py (500 $\mu$ g./l.) + lysine	104.0	4.4	5.9	17.4	16.4

estimated by Dr. D. T. Hughes at Oxford) had been achieved. In the deficient cells grown without added lysine (Exp I), there was a marked increase in diaminopimelic decarboxylase activity (measured with added pyridoxine). The presence of lysine in the growth medium produced a reduction in diaminopimelic decarboxylase activity (Expts. I and II, IV and V), particularly under pyridoxine deficiency, when the effect was noticeable on activities measured both with and without added pyridoxine.

These experiments indicate that diaminopimelic decarboxylase is an indispensable enzyme concerned with lysine biosynthesis, since (1) pyridoxine deficiency did not lower the levels of coenzyme-bound diaminopimelic decarboxylase unless lysine was provided in the growth medium; (2) under conditions of stress caused by deficiencies of both pyridoxine and lysine, there can be adaptation to greater production of diaminopimelic decarboxylase apoenzyme.

*Distribution of diaminopimelic decarboxylase.*

A survey of the distribution of diaminopimelic decarboxylase in microorganisms is being carried out, with the primary object of finding possible correlations in enzyme activity with the cellular diaminopimelic acid contents, nutritional requirements, or biochemical characteristics. Ultimately we hope to get more information on the course of lysine biosynthesis in bacteria.

TABLE 6

DISTRIBUTION OF DIAMINOPIMELIC DECARBOXYLASE IN MICROORGANISMS

Organism	No. of strains tested	DAP content	DAP decarboxylase $Q_{CO_2}$	Lysine requirement for growth
<i>Aerobacter aerogenes</i>	29	+	6-12	0
<i>Klebsiella pneumoniae</i>	2	+	0.4 & 24	0
<i>E. coli</i>	36	+	0-18	0
<i>Proteus vulgaris</i>	1	+	0	0
<i>Pseudomonas aeruginosa</i>	1	+	1.1	0
<i>Bacillus subtilis</i>	1	+	0	0
<i>Rhodopseudomonas spheroides</i>	1	++	0.6	0
<i>Rhodospirillum rubrum</i>	1	+	1.2	0
<i>Micrococcus lysodeikticus</i>	1	0	3.8	0
<i>Sarcina lutea</i>	1	0	5.8	0
<i>Staphylococcus aureus</i>	1	0	2.9	0
<i>Streptococcus faecalis</i>	2	0	0	+
<i>Leuconostoc mesenteroides</i>	1	0	0	+
<i>Lactobacillus arabinosus</i>	2	++	0	+
<i>Actinomyces</i> sp.	1	0	3.8	0
Yeast (baker's)	1	0	0	0
<i>Neurospora crassa</i>	1	0	0	0
<i>Chlorella pyrenoidosa</i>	1	0	0	0

Sixty-seven strains of Enterobacteriaceae were examined. They showed great and unexplainable variations in decarboxylase content (Table 6),  $Q_{CO_2}$  values between 0 and 18 being obtained with different strains of *E. coli* (31). Owing to the insensitivity of the Warburg technique for measuring low activities, we could not estimate levels of enzyme below a  $Q_{CO_2}$  of 0.4, therefore a value of 0 only means that the enzyme activity was below this figure and not



necessarily that the enzyme was absent from the organism. In fact, Davis (unpub.), using a more sensitive microbiological technique, has been able to detect decarboxylase activity in strains where we could not.\* Also, because of variability in effect of growth time on the decarboxylase activities of different strains, it is possible that the cells were not always harvested at the optimal time for the particular organism. It is felt, therefore, that no undue significance should be placed on the apparent absence of decarboxylase from one strain or species of an organism. When it is absent from *all* the strains examined under a variety of growth conditions and tested by two methods, more reliance can be placed on the result.

Relatively few organisms outside the Enterobacteriaceae have been examined. One interesting finding is the occurrence of diamino-pimelic decarboxylase in three of the less exacting members of the gram-positive cocci (*M. lysodiekcticus*, *Sarcina lutea*, and *Staphylococcus aureus*), none of which contain cellular diaminopimelic acid. The levels of enzyme were of the same order as those found in many strains of *E. coli*. In view of the very high specificity of diaminopimelic decarboxylase, it does not seem likely that the enzyme would have another natural substrate, and one can only imagine that the function of the enzyme in these organisms, as in *E. coli*, is to produce lysine. Thus it appears likely that in these cocci and in *E. coli*, the same biosynthetic route to lysine is used; in the cocci diaminopimelic acid must be only a transient intermediate, since it was not found even in the soluble amino acids of *S. lutea*. The strain of *S. lutea* in use grew well on solid media containing, as the sole source of carbon and nitrogen, any one of the common amino acids, including diaminopimelic acid, but excluding glutamic acid and  $\alpha$ -aminodipic acid. Diaminopimelic acid, but not lysine, was found to stimulate oxygen uptake by cell suspensions of *M. lysodeikticus* but not of *S. lutea*. These observations are not necessarily of significance in lysine metabolism, since diaminopimelic acid is oxidized by the L-amino acid oxidase of *Neurospora* (18), but can

\* Davis has confirmed our observation on the lack of decarboxylase in the three lysine-requiring mutants.



play no part in lysine metabolism in this organism, where all the lysine is known to be derived from  $\alpha$ -aminoadipic acid (92).

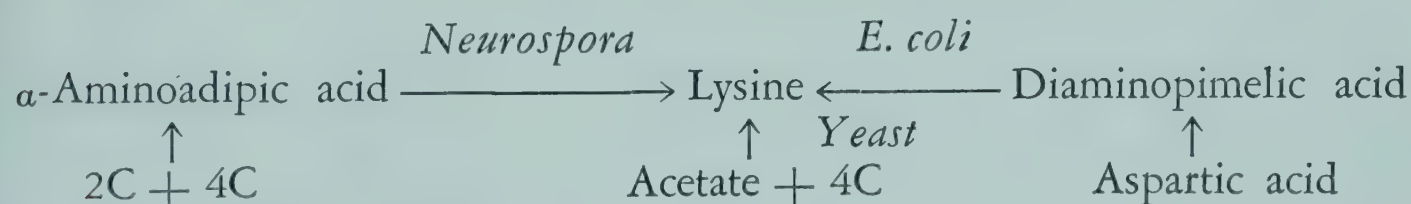
Table 6 shows that there is no relation between the distribution of diaminopimelic acid and that of its decarboxylase. Another organism, besides the three already mentioned, which contained diaminopimelic decarboxylase but not the amino acid, was *Actinomyces*. Here the methyl homologue of diaminopimelic acid was present, but this amino acid is not a substrate for the decarboxylase (96).

Three lysine-requiring organisms had no diaminopimelic decarboxylase activity, although the *Lactobacillus* had a high level of diaminopimelic acid. It is not yet possible to say whether the lysine requirement is due specifically to this apparent absence of decarboxylase. Diaminopimelic acid does not substitute for lysine as a growth factor for these organisms (54, 101).

The three non-bacterial organisms examined contained no diaminopimelic acid decarboxylase. In *Neurospora*, diaminopimelic acid is not a growth factor for lysine-requiring mutants (58, Mitchell, pers. commun.)

### SUMMARY

Our scanty knowledge regarding the comparative aspects of lysine biogenesis can be summarized with one last scheme.



This divergence of lysine from the usual pattern taken by amino acids is probably due to its 6-carbon chain, which renders it liable to ring closure. The two patterns of lysine metabolism which have been dealt with here are both means of overcoming this tendency to ring closure. Possibly the one adopted by molds and mammals is that of blocking the amino group by acetylation—a reaction used also in ornithine biosynthesis where the same problem has to be overcome. A method of avoiding ring closure adopted by bacteria is to synthesize



a 7-carbon compound and then to decarboxylate it. The majority of bacteria also utilize this 7-carbon amino acid as a structural component of the cell.

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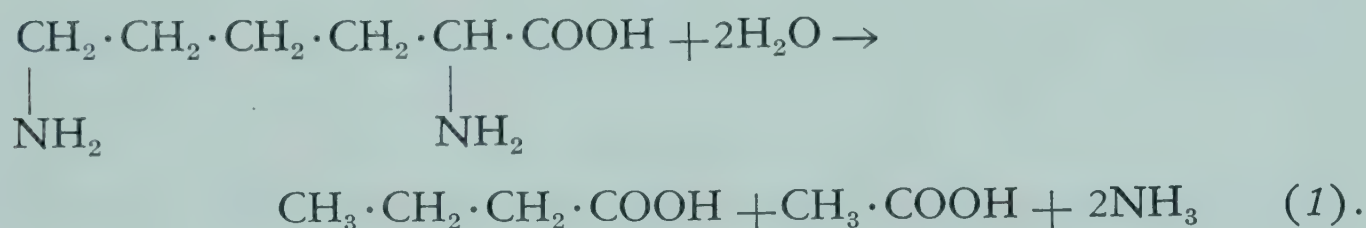
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# ON THE ANAEROBIC DEGRADATION OF LYSINE

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AN AMINO-ACID-FERMENTING *Clostridium*, strain HF (1, 2), and a mixed culture of two *Escherichia coli* strains (3) have been found to convert lysine to butyric acid, acetic acid, and ammonia, according to the reaction:



This overall conversion involves no net oxidation or reduction and thus does not fall into that class of coupled oxidation-reduction reactions between pairs of amino acids known as Stickland reactions. Hence it is surprising that the amino acid pair, ornithine plus lysine, supports the growth of *Clostridium* HF as well as the pair ornithine plus proline, since the latter pair undergoes a typical Stickland reaction. In both cases the ornithine is oxidized to acetate, carbon dioxide, and ammonia, but only the proline undergoes a net reduction—to  $\delta$ -aminovalerate. The ability of lysine to substitute for proline in these fermentations is as yet unexplained.

An examination of the products of a fermentation of ornithine and 2- $\text{C}^{14}$ -lysine revealed that both the acetate and butyrate contained  $\text{C}^{14}$ . Stepwise degradation of these compounds, after suitable dilution with carrier acids, showed the isotope to be located in the methyl carbon atom of acetate and carbon atoms 2 and 4 of butyrate (Table 1). The high degree of labeling of carbon atom 2 of butyrate as compared to carbon atom 4 suggested that much of the butyrate might have been derived directly from the lysine carbon skeleton rather than by resynthesis from  $\text{C}_2$  pieces. Support for this



TABLE 1

DISTRIBUTION OF ISOTOPE IN ACETATE AND BUTYRATE  
DERIVED FROM ORNITHINE PLUS 2-C<sup>14</sup>-LYSINE FERMENTATION

ACETATE		BUTYRATE			
CH <sub>3</sub> -	-COOH	CH <sub>3</sub> -	CH <sub>2</sub> -	CH <sub>2</sub> -	-COOH
10.9	0.6	18.4	0.3	89.7	0.3

The isotope distribution in the fatty acids is expressed as cts. per min. per mg.  $\text{BaCO}_3$ . Recoveries were checked by total wet combustions on each acid.

hypothesis was obtained by examination of the fatty acids formed when 2-C<sup>14</sup>-lysine was decomposed anaerobically by dried cells of *Clostridium* HF (Table 2). Under these conditions the butyrate is

TABLE 2

ISOTOPE DISTRIBUTION IN PRODUCTS DERIVED FROM 2-C<sup>14</sup>- AND 6-C<sup>14</sup>-LYSINE

SUBSTRATE	PRODUCTS					
	Acetate CH <sub>3</sub> -COOH		Butyrate CH <sub>3</sub> -CH <sub>2</sub> -CH <sub>2</sub> -COOH			
2-C <sup>14</sup> -DL-Lysine						
CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·C <sup>14</sup> H·COOH <div style="text-align: center;">                             NH<sub>2</sub>                      NH<sub>2</sub></div>	23.8	0.2	0.7	21.4	0.4	
6-C <sup>14</sup> -DL-Lysine						
C <sup>14</sup> H <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·CH·COOH <div style="text-align: center;">                             NH<sub>2</sub>                      NH<sub>2</sub></div>	13.3	0	112.6	0.4	0	0

Specific activities of each carbon are cts./min./mg.  $\text{BaCO}_3$  after addition of carrier fatty acid.

labeled exclusively in carbon atom 2 and no recombination of labeled acetyl derivatives with subsequent reduction to butyrate appears to have occurred.

Decomposition of 6-C<sup>14</sup>-lysine under similar conditions also gives rise to methyl-labeled acetate, but the butyrate now is labeled only in carbon atom 4 (Table 2). These results suggest that cleavage of a C<sub>2</sub> piece may occur from either end of the molecule, so as to leave a C<sub>4</sub> piece labeled as indicated.

The intermediary role of  $\alpha$ -aminoadipic acid, for which there is some evidence in mammalian and *Neurospora* systems, appears unlikely here, since it would necessitate oxidation of carbon atom 6 of lysine to a carboxyl group, followed by reduction again to a methyl carbon. Also,  $\alpha$ -aminoadipic acid, as a free compound, is inert in the system. Likewise, the dihexenoic acid, sorbic acid, does not occur as a free intermediate in the clostridial system, although it fits the requirements imposed by the isotope data.

Soluble extracts from certain lots of dried cells were found to be incapable of producing labeled butyrate from 2- $C^{14}$ -lysine but did form labeled acetate. In parallel samples containing 6- $C^{14}$ -lysine, no isotope was found in either the volatile or non-volatile acid fractions, but instead a compound or compounds in the neutral volatile fraction contained  $C^{14}$ . The isotope was detected in the  $CO_2$  derived from the neutral volatile fraction after treatment with the Van Slyke-Folch oxidizing mixture. The identity of the product or products in this fraction, which should contain carbons 3-6 of lysine, is being investigated. It may be a precursor of butyrate in the system or, alternatively, a product of a side reaction undergone by the actual intermediate.

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# THE ISOLATION AND METABOLISM OF THE $\alpha$ -KETO ACID OF LYSINE \*

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THE DISCOVERY of pipecolic acid as a naturally occurring constituent of plant extracts (1, 2) has led to studies of its metabolic origin.  $C^{14}$ -labeled lysine has been shown to be converted to pipecolic acid in plants (3, 4), animals (5), and more recently in *Neurospora* (6, 7). It has been suggested that the  $\alpha$ -keto acid of lysine in its cyclized form is the intermediate in this conversion (8, 9, 10). This compound has been isolated by Meister (10) and has been shown to be the product when lysine is oxidized by the L-amino acid oxidase of *Neurospora* (6). Evidence is presented here that radioactive  $\alpha$ -keto- $\epsilon$ -aminocaproic acid exists in solution in its cyclized form<sup>1</sup> and is converted by *Neurospora* to pipecolic acid and also back to lysine.

## EXPERIMENTAL

*Preparation of  $\alpha$ -Keto- $\epsilon$ -aminocaproic Acid.* *Neurospora* wild-type strain 4A was grown for three weeks as described by Thayer and Horowitz (11). The medium from 6 Fernbach flasks was combined and the L-amino acid oxidase precipitated with ammonium sulfate, resuspended in phosphate buffer, and dialyzed as described by these authors. Determinations of enzyme activity in the Warburg apparatus indicated that the total amount of enzyme was sufficient to oxidize

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<sup>1</sup> The cyclized form will be referred to as  $\Delta^1$ -dehydropipecolic acid. The basis for this designation is given in the text. See also (10).

100 mg. of lysine to completion in 2 to 3 hours. This amount of carboxyl- $C^{14}$ -labeled lysine with a specific activity of 7500 counts per minutes per mg., plus the enzyme, was dissolved to make a final volume of 100 ml. in 0.05 *M*. phosphate buffer, pH 6.0. The mixture was shaken for 4 hours in an oxygen atmosphere. It was then dialyzed for 24 hours in the cold against 3 one-liter portions of distilled water. The dialysate was concentrated in vacuo, yielding

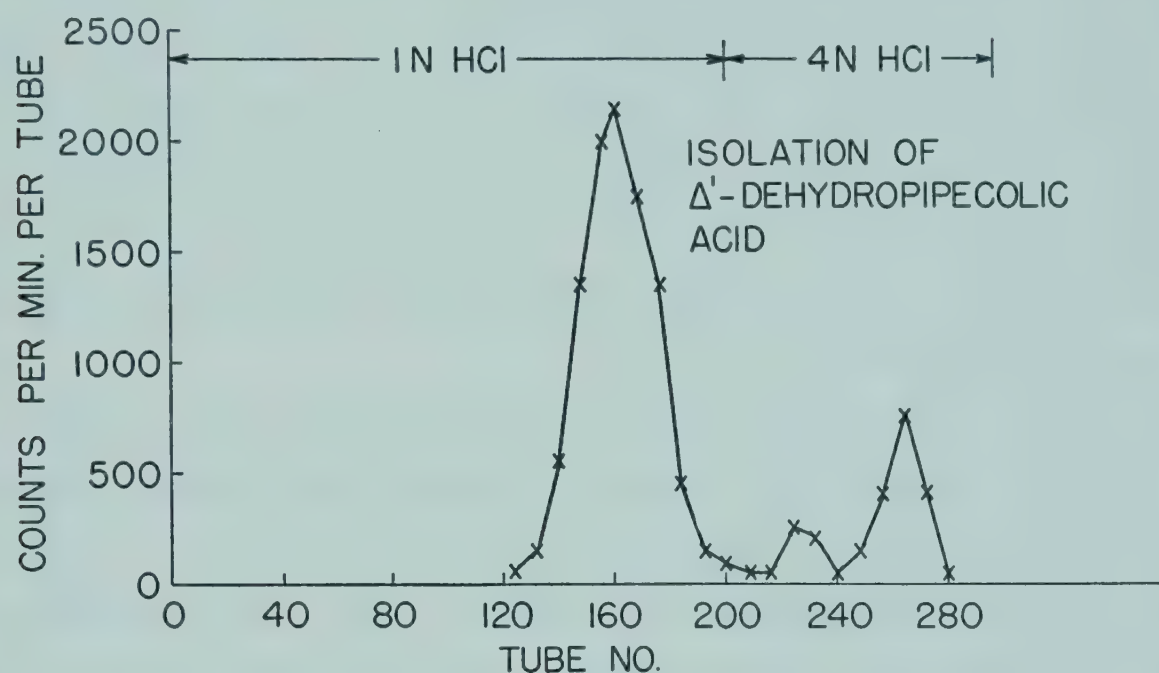


FIG. 1. Isolation of a  $\Delta^1$ -dehydropipecolic acid (DHP). Chromatographed on Amberlite IR-112,  $2.5 \times 50$  cm. column, and eluted with the acid strengths shown. Each tube contained 5 ml.

75 per cent of the original radioactivity. For desalting, the concentrated dialysate was adsorbed on Dowex-50 (hydrogen form), which was then washed with distilled water, and the radioactive compounds eluted with 2 *M*. ammonia. The ammonia was removed in vacuo. An aliquot containing 300,000 counts per minute was then fractionated on Amberlite IR-112<sup>2</sup> with hydrochloric acid (Fig. 1). The first peak was taken to dryness several times to remove hydrochloric acid. A total yield of 80,000 counts per minute was obtained. In addition, about 8,000 counts of residual lysine were recovered from the next peak. The overall yield was about 20 per cent. Paper

<sup>2</sup> This resin is described (Rohm and Haas Company) as a strong cation exchanger of high porosity. It was tested when attempts to elute the compound from Dowex-50 with hydrochloric acid and with buffers were unsuccessful.



chromatograms of the isolated material were run in three solvents<sup>3</sup> and showed the presence of  $\Delta^1$ -dehydropipecolic acid (DHP) plus some non-radioactive valine.

The preparation was further checked for the presence of radioactive lysine or pipecolic acid by carrier recrystallization. A total of 5000 counts of dehydropipecolic acid was mixed with 300 mg. of non-radioactive DL-pipecolic acid hydrochloride, and the pipecolic acid was crystallized from methanol-acetone (2). After three crystallizations, the pipecolic acid did not contain any radioactivity. Similarly, non-radioactive lysine was added to a duplicate aliquot, converted to the L-lysine monopicrate (12), and recrystallized from hot water. The lysine picrate, m. p. 264-266° (uncorr.), also did not contain any radioactivity. It is estimated that these methods would detect a one per cent impurity.

While the preparation of this compound by means of the *Neurospora* oxidase has been of interest in connection with lysine metabolism in this organism, it is clear that as a synthetic procedure, the method is poor. The method of Meister (10), using snake venom oxidase, is preferable. More recently, we have observed non-enzymatic transamination of lysine with glyoxylic acid, as described by Snell (13). A small amount of dehydropipecolic acid was formed. It is possible that further development of this method may yield a good synthetic procedure.

*Properties of the Isolated Compound.* The product isolated by Meister (10) and identified as  $\alpha$ -keto- $\epsilon$ -aminocaproic acid, formed a phenylhydrazone *slowly*, was decarboxylated by hydrogen peroxide with the formation of  $\delta$ -aminovaleric acid, reacted with *ortho*-aminobenzaldehyde, and was catalytically hydrogenated to pipecolic acid. The same properties were also exhibited by the compound isolated in these experiments. The last two properties are indicative of a cyclized structure.

The identity of the isolated compound with authentic  $\alpha$ -keto- $\epsilon$ -

<sup>3</sup> The solvents used were butanol-acetic-acid-water (11 to 3 to 4 parts by volume), pyridine-water (6.5 to 3.5 parts by volume), and phenol saturated with water.



amino-caproic acid<sup>4</sup> has been confirmed by paper chromatography. Both compounds migrate with mobilities ( $R_f$ ) of 0.52, 0.79, and 0.92 in butanol-acetic, pyridine-water, and phenol, respectively. The compounds were located by radioactivity or by spraying with PDAB.<sup>5</sup>

The evidence that the compound exists mainly in the cyclized form is given below.

1. No separation into two compounds has been observed on paper chromatograms, ion exchange columns, or paper electrophoresis.

2. The compound which is present gave only a faint yellow color with ninhydrin on paper chromatograms (particularly in acid solvents). The open-chain keto acid would be expected to react, due to the free  $\epsilon$ -amino group. The phenylhydrazone of DHP does give a purple color on paper. Phenylhydrazine itself does not.

3. The compound had an  $R_f$  on paper chromatograms, ion exchange columns, and paper electrophoresis (pH 7.0) which was close to that of pipecolic acid and proline. In 5 *N* acetic acid (paper electrophoresis) the compound was more acidic than pipecolic acid.

4. Catalytic hydrogenation at atmospheric pressure (14) produced DL-pipecolic acid in 75 per cent yield based on the original radioactivity. The residual material was unreacted compound and an insoluble residue. Evidence for racemic pipecolic acid is shown in Table 1. The D-pipecolic acid-D-tartrate contained one half of the total radioactivity.

All these observations are compatible with a cyclized structure, in equilibrium with a small amount of the open-chain keto acid. The designation of the double bond as  $\Delta^1$ - is less certain, since tautomerism would be expected. Other properties are suggestive of ready polymerization. The compound was not eluted from several ion exchange resins with acid. It was adsorbed on Norit at 85° C., and eluted in 50 per cent yield with pyridine-water mixtures. The

<sup>4</sup> Kindly furnished by Dr. Meister.

<sup>5</sup> The chromatograms were sprayed with 2 per cent *p*-dimethylaminobenzaldehyde in pyridine. An orange color was developed by heating for 2 to 3 minutes at 105° C. Amino acids gave yellow colors. The reagent will detect 10 $\gamma$  of dehydropipecolic acid. The color intensifies after 16 hours standing at room temperature.



TABLE 1

RESOLUTION OF RADIOACTIVE PIPECOLIC ACID FORMED BY HYDROGENATION  
OF DEHYDROPIPECOLIC ACID

Fraction	Yield *	Specific activity	Total activity
	mg.	counts per min. per mg.	counts per min.
DL-pipecolic acid plus D-tartaric acid	500	5.2 **	2600
D-pipecolic acid- D-tartrate	310	5.3	
Second crystallization	200	5.0	1250

\* All values are based on pipecolic acid hydrochloride. Experimental details are given in the text.

\*\* This value is calculated from the original total activity.

compound was unstable to heat in 2 *N* hydrochloric acid. These properties would be expected of the  $\Delta^1$ - or  $\Delta^2$ -structure (15), and more direct chemical work will be required to resolve these questions.

*Metabolism of Dehydropipecolic Acid.* To study the utilization of DHP by *Neurospora*, lysineless strain 4545 was grown in the presence of radioactive DHP plus non-radioactive lysine and pipecolic acid. Each flask contained 2 mg. of L-lysine, 3 mg. of L-pipecolic acid, and 2 mg. of radioactive DHP containing 7500 counts per minute per mg., plus basal medium. A total of 4 flasks were incubated for 5 days. Some inhibition of growth was noted during the first 24 hours, and the final dry weight of mycelium was 50 mg. per flask, compared to the usual 60-70 mg.

Three fractions were separated: the medium, a boiled extract of the dried mycelium (NPN), and the protein. The protein fraction was washed with trichloroacetic acid, sodium hydroxide, acetone, and ether. The full details of these experiments are omitted, since they were the same as those already described for experiment starting with C<sup>14</sup>-labeled lysine (7).

The amount of radioactivity found in the medium, NPN, and protein fractions, respectively, was 37,000, 1600, and 1100 counts per minute. The experiment was started with 60,000 counts per

minute of DHP. Most of the radioactivity of the medium was starting material. The small percentage of counts found inside the cell differs greatly from results obtained with radioactive lysine or pipercolic acid (7). The most probable explanation is that DHP does not readily penetrate the cells. Some DHP may be decarboxylated and thus lose the label.

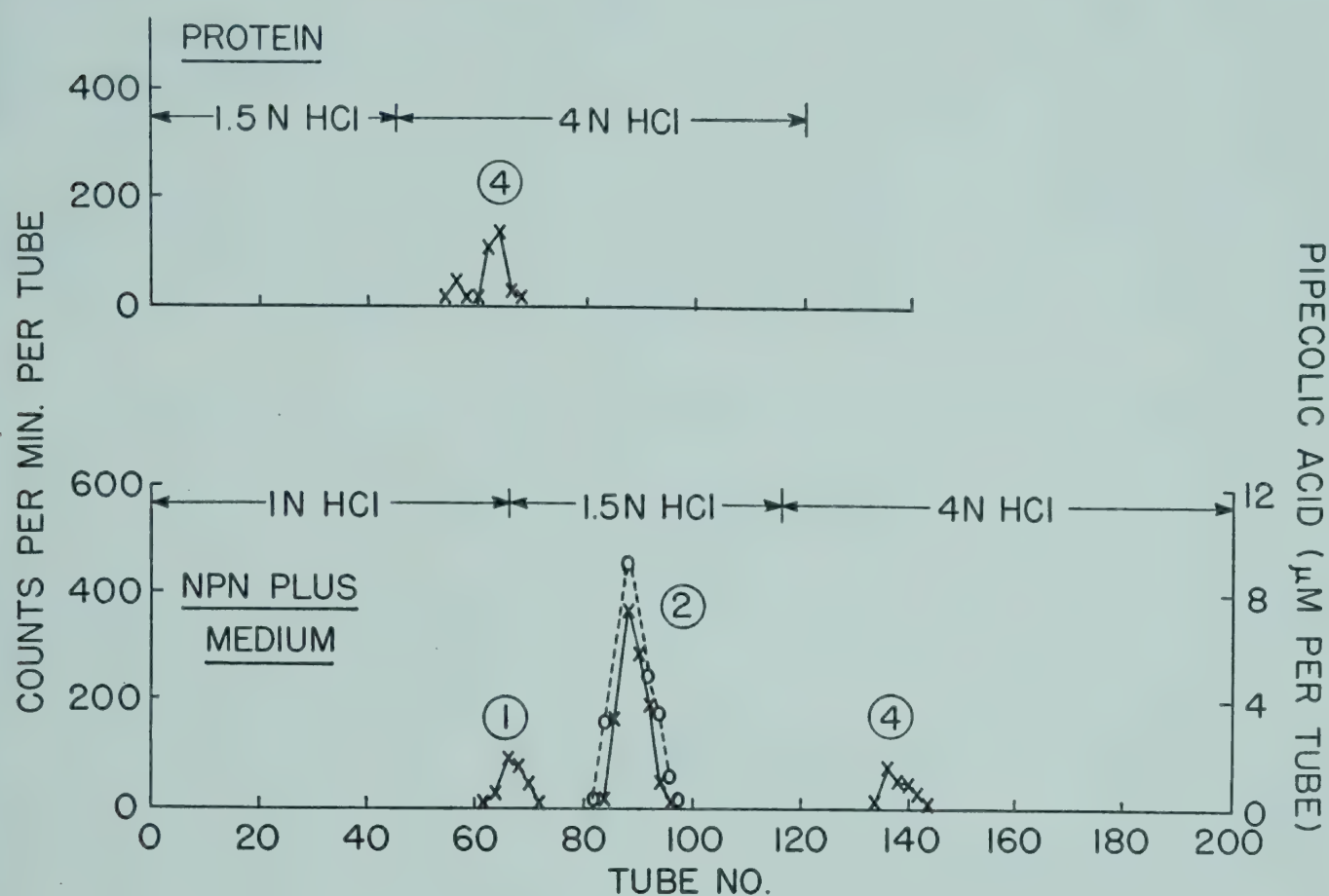


FIG. 2. Chromatography of cationic fractions on Dowex-50. A  $2.5 \times 50$  cm. column was used and eluted with the acid strengths shown. Each tube contained 5 ml. (NPN) or 6.5 ml. (Protein). The solid lines are radioactivity determinations (left ordinate) and the dashed lines are pipecolic acid concentrations (right ordinate).

The medium and NPN were combined because of the small amount of radioactivity. This fraction was adsorbed on Dowex-50, and salts and *anionic* compounds were removed with water. The *cationic* constituents were eluted with ammonia (7). The ammonia-free eluate contained 30,000 counts per minute. This was treated with Norit at  $85^{\circ}$  C. for 5 minutes to remove DHP.<sup>6</sup> Approximately 80 per cent of the radioactivity of the *cationic* fraction (NPN plus medium) was removed by Norit treatment. The remainder of this

<sup>6</sup> If the Norit treatment is omitted, small amounts of radioactive dehydropipecolic acid are eluted from the column along with the other compounds.



fraction and also the hydrolyzed protein were each chromatographed on Dowex-50 (hydrogen form). The results are shown in Fig. 2.

The fractions of each peak were analyzed for pipecolic acid (16). In Peak 2 of the NPN plus medium the amount of pipecolic acid in each tube was proportional to the radioactivity. The protein hydrolysate showed no radioactive peak in this region. The compound in this peak was further identified as radioactive pipecolic acid by chromatography in three solvents, red fluorescence after ninhydrin spray (2), and carrier recrystallization with authentic material (Table 2). This material has not yet been resolved, but only the L-isomer is expected to be radioactive, since this was true for pipecolic acid isolated after growth on radioactive lysine (7).

TABLE 2

CARRIER RECRYSTALLIZATION OF PIPECOLIC ACID ISOLATED AFTER GROWTH ON DEHYDROPIPECOLIC ACID

Fraction	Yield	Specific activity	Total activity *
	mg.	counts per min. per mg.	counts per min.
First crystallization	87.0	6.1	
Second crystallization	49.0	5.3	
Third crystallization	36.0	5.5	1100

\* The isolated pipecolic acid peak containing 1700 counts per minute was combined with 200 mg. of DL-pipecolic acid hydrochloride and crystallized from methanolacetone. The final total activity is based on 200 mg.

An asymmetric reduction of DHP is postulated for the enzyme-catalyzed reaction. The compound in Peak 1 has not been identified, but a radioactive peak emerges in the same region in experiments starting with  $C^{14}$ -lysine. Peak 4 in both fractions has been identified as radioactive lysine by chromatography, paper electrophoresis, and carrier recrystallization (Table 3).

In addition to these radioactive peaks, the *anionic* fraction was radioactive. The major component of this fraction was shown by paper electrophoresis to be the same as a compound found in experiments starting with  $C^{14}$ -lysine. This compound yields  $\alpha$ -hydroxy- $\epsilon$ -aminocaproic acid upon acid hydrolysis (7), and more recently has

TABLE 3

CARRIER RECRYSTALLIZATION OF LYSINE ISOLATED AFTER GROWTH  
ON DEHYDROPIPECOLIC ACID \*

Fraction	Yield	Specific activity	Total activity
	mg.	counts per min. per mg.	counts per min.
Lysine monopicrate	228	2.0 **	460
First crystallization	69.3	1.9	
Second crystallization	46.5	2.1	

\* The lysine peak isolated from the protein containing 460 counts per minute was combined with 100 mg. of L-lysine hydrochloride. This was converted to the monopicrate and recrystallized from hot water.

\*\* This value is calculated from the original total activity.

been tentatively identified as  $\alpha$ -hydroxy- $\epsilon$ -N-acetylaminocaproic acid (based on comparison with synthetic material).<sup>7</sup>

The pipecolic acid isolated contained 1700 counts per minute, which was 4.6 per cent of the DHP *metabolized* (total original radioactivity minus DHP remaining). The lysine isolated contained 710 counts per minute, which was 1.9 per cent, while the  $\alpha$ -hydroxy- $\epsilon$ -N-acetylaminocaproic acid contained 2.6 per cent of the radioactivity which was utilized. These values indicate a very small conversion, which is probably due to the slow penetration of DHP into the cell. The appearance in these experiments of radioactive metabolites which are the same as those formed from lysine, and the fact that the relative amounts of these follow the pattern from lysine (7) favors DHP as the physiological intermediate between lysine and pipecolic acid. A further point here is the ability of the L-oxidase of *Neurospora* to oxidize lysine to DHP at a rate (about two-thirds that of leucine) which is compatible with that of lysine utilization.

The conversion of DHP to lysine offers little evidence on the question of lysine transamination, since it is possible that the conversion involves a  $\Delta^1$ - to  $\Delta^6$ - switch of the double bond. The latter compound could open to aminoadipic semialdehyde (or a derivative), which is probably on the biosynthetic pathway between amino-

<sup>7</sup> Schweet, R., Holden, J., and Lowy, P., unpub.



adipic acid and lysine. The rate of conversion of DHP to lysine which has been observed in these experiments would not be sufficient to satisfy the growth requirements of *lysineless* strains. This was found to be the case in several preliminary experiments. DHP did not stimulate growth in the presence of limiting amounts of lysine, even of strains which can utilize D-lysine for growth (17).<sup>8</sup> The conversion of D- to L-lysine by these strains may go through internally produced DHP as intermediate, although other possibilities exist. It is clear that evaluation of the physiological rate and mechanism of the DHP to lysine conversion requires the development of in vitro systems which will catalyze these reactions.

#### SUMMARY

1. The  $\alpha$ -keto acid of lysine was formed when L-lysine was oxidized by *Neurospora* L-amino acid oxidase.
2. This compound was present in solution mainly as the cyclized form,  $\Delta^1$ -dehydropipecolic acid. The location of the double bond is considered tentative.
3. Radioactive dehydropipecolic acid was converted to pipecolic acid and back to lysine by *Neurospora* in vivo.
4. Two other radioactive metabolites were formed from dehydropipecolic acid. These were also formed from radioactive lysine.
5. The evidence which points to  $\Delta^1$ -dehydropipecolic acid as the physiological intermediate between lysine and pipecolic acid is discussed.

#### ACKNOWLEDGMENTS

The authors wish to express appreciation to Drs. Henry Borsook and Norman Horowitz for their generous support and encouragement of this work. We are indebted to Dr. H. K. Mitchell for a number of helpful discussions. Mr. R. J. Busser has rendered valuable technical assistance.

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## DISCUSSION

DR. DAVIS: Diaminopimelic acid (DAP) seems to have received only limited acceptance as an intermediate in lysine biosynthesis because of the fact that mutants blocked before DAP showed a relative requirement for lysine in addition to their absolute requirement for DAP. These results seemed to imply that external DAP got incorporated into cellular DAP more rapidly than into lysine; and at the time when this work was published this aspect of the problem was still obscure. Nevertheless, I could not escape the conclusion, on the basis of the enzymatic evidence that Dr. Work has presented, that DAP is squarely on the path to lysine. This decision was based on the conviction, which I have elaborated elsewhere in this symposium (in the discussion on isoleucine biosynthesis), that the most decisive evidence presently available that a compound is a true intermediate in a microorganism is precisely this kind of enzymatic evidence: namely, that an enzyme that forms or one that utilizes the compound is present in the wild type but is absent from a mutant blocked in the biosynthetic path under consideration. (This evidence must be supplemented, of course, by the demonstration that the compound *can* be converted to the product(s) of that path.)

More recently, the obscure aspects of the DAP-lysine problem seem to me to have become quite clear through consideration of three facts: that permeability (i. e., accessibility) barriers not only are theoretically possible but are very real; that the amount of DAP present (in bound form) in *E. coli* is only a fraction—roughly  $\frac{1}{4}$ —of the amount of lysine present; and



that the addition of an amino acid has been shown in many cases by the Carnegie group to inhibit completely endogenous synthesis of that amino acid. The behavior of the mutants blocked before DAP can now be entirely explained if one assumes that added DAP penetrates the cell slowly; that endogenous and/or exogenous DAP is the sole source of cellular DAP and lysine (in the absence of added lysine); and that added lysine penetrates freely and can serve as the source of cellular lysine but not of DAP. It would then be expected that in a mutant blocked before DAP the addition of lysine would decrease the DAP requirement about 5 times. Hence, if the rate of penetration of DAP were growth-limiting, and if this penetration were not affected by addition of lysine, this addition would increase the growth rate about 5 times. This is essentially the effect observed.

However, one might have reservations about this *ad hoc* assumption of a permeability barrier. Fortunately, though, this is one of the few cases where positive evidence for such a barrier exists. For the Carnegie group, in their isotopic competition studies, found that added DAP did not get incorporated into lysine. When I first heard of this finding, it made the case for DAP as a precursor of lysine look even weaker—until I later learned that added DAP didn't get incorporated into cellular DAP either! Since each of the other amino acids in the cellular protein is readily formed from the free amino acid (as shown by the incorporation of labeled material), and since pools of free amino acids exist in many bacterial species, I would be willing to assume that on minimal medium fixed cellular DAP, like all fixed amino acids, necessarily arises from the corresponding free amino acid. The failure of *E. coli* to incorporate added DAP would then imply that added DAP does not get into the wild-type cell fast enough to compete effectively with endogenous DAP. It is therefore not surprising that added DAP does not get into the mutant cell fast enough to support rapid growth.

Part IV

*METABOLISM OF METHIONINE, CYSTEINE  
AND THREONINE*





# SYNTHETIC PATHWAYS OF METHIONINE, CYSTEINE, AND THREONINE \*

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## INTRODUCTION

IN 1960 CYSTINE will celebrate the 150th anniversary of its discovery by Wollaston. During this period cystine has passed through the stages of being an essential amino acid, then a non-essential amino acid, and it now enjoys the status of an amino acid which "spares" the requirement for the essential amino acid, methionine, in the diet of animals. The discovery by Jackson and Block, in 1932, that methionine will replace cystine in the diet of the rat elevated methionine to the position of an essential amino acid. Recently, however, it has been conceded that a portion of the methionine molecule, its methyl group, is not entirely essential in the diet, but can be presumed to be semi-essential under certain conditions.

Threonine still holds the position of an essential amino acid in the diet of animals, including man, although its total synthesis in certain organisms appears to be well established.

These biochemical events suggest the conclusion that "established facts" are subject to reinterpretation as more information becomes available in the course of further exploration. The data to be reviewed here should be looked upon as exploratory, with the emphasis on the conditions under which they were obtained. With this understanding in mind, it will be perhaps an easier task to evaluate individual data and to reconcile apparent inconsistencies in others.

\* All published and unpublished data from this laboratory referred to in this paper were obtained through the support by grants from the National Cancer Institute, U. S. Public Health Service, Bethesda, and from the U. S. Atomic Energy Commission. All isotopic materials were obtained on authorization from the Atomic Energy Commission.



The general scheme illustrating the metabolic interrelationships in the synthesis of methionine, cysteine, and threonine is shown in Fig. 1. In the center of Fig. 1 a compound marked "X" represents an unknown intermediate which apparently arises from homocysteine and a one-carbon compound,  $C_1$ , which in turn originates from a host of metabolites by various routes. This compound "X" is an

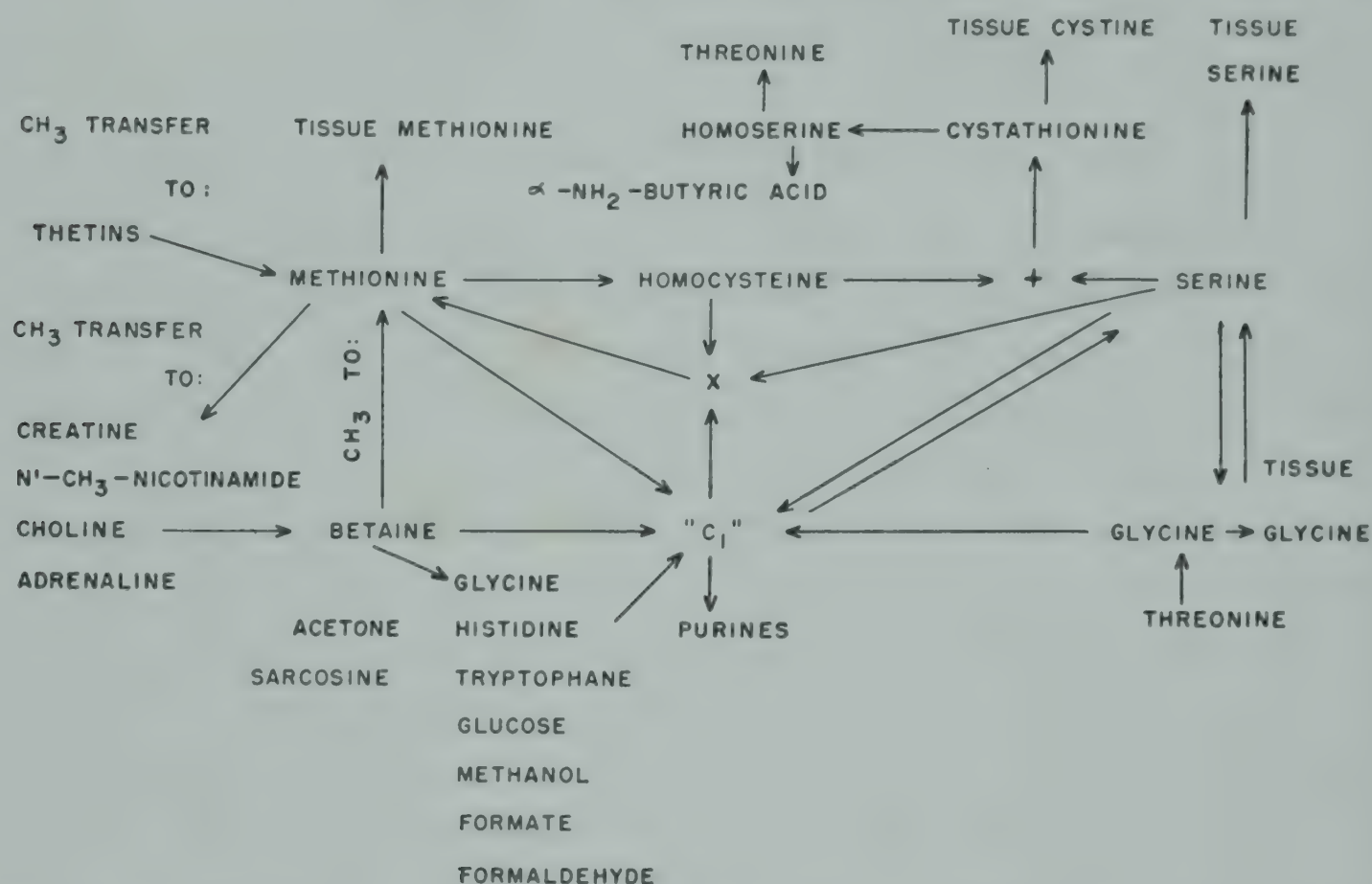


FIG. 1. General interrelationships in the synthesis of methionine, cystine, and threonine.

intermediate in the synthesis of methionine and is a carrier of the potential methyl group of methionine. The possible mechanism of the synthesis de novo of the methionine methyl group has been previously discussed in these symposia (1), and I shall present some additional information obtained in our laboratory on the subject.

The methyl group of methionine can also originate by direct transfer from such metabolites as betaine and the thetins. Some aspects of the mechanism of methionine synthesis by this route have been discussed previously (1) with some stated omissions. Some additional information on the subject will be reviewed briefly.

It is clear from Fig. 1 that in studies involving whole animals, whether the technique employed is nutritional or tracer, the evalua-

tion of the extent of synthesis of methionine, cysteine, or threonine from various precursors must take into account the relationship of the synthesis of these amino acids to other metabolic reactions and metabolite concentrations, which will obviously play an important role in determining the extent of this synthesis. Perhaps similar considerations apply to studies in which whole homogenates of organs or even "purified" systems are employed. In addition, the question of the relative availability of the metabolites to the enzymatic action in whole animals, slices, homogenates, and "purified systems" calls for careful evaluation of the data within the limitations of the conditions under which they were obtained. The elasticity of the metabolic net and the variety of the possible approaches in the studies of its component threads are such that equivocal and unequivocal answers are possible as exploration continues. The correct answer appears to be that one which is supported by the experimental evidence available at the time. A fact is as reliable as the method employed to obtain it. No claim, therefore, is made in the present review that the facts as we know them today will withstand the test of time and further study.

I leave it to Dr. Black to cover the highlights of the synthetic pathways of threonine, to which he has contributed.

### SYNTHESIS OF CYSTEINE

Certain aspects of the synthesis of methionine *in vivo* have arisen from the studies of the synthesis of cysteine from methionine. We shall, therefore, review first the work done on the synthetic pathways of cysteine.

In 1932, Jackson and Block (2) announced that methionine will replace cystine in the diet of the rat. This was the first indication of the synthesis of cysteine from methionine *in vivo*. These workers suggested several lines of further research which could be followed in establishing the conversion of methionine sulfur to that of cysteine, namely, the formation of cystine from methionine in cystinurics, the augmentation of the excretion of mercapturic acids on feeding methionine together with various aromatic compounds, the feeding



to animals of diets containing methionine as the sole sulfur-amino acid, etc. These suggestions were followed up in several laboratories. Feeding of methionine to cystinurics augmented the excretion of cystine in the urine (3); feeding of methionine together with halogenated benzenes increased the formation of the corresponding mercapturic acids (4); and methionine, fed as the sole sulfur-amino acid in the diet of the rat, supported growth (5). Either optical isomer of methionine was effective in the apparent synthesis of cysteine (6), a fact indicating the inversion *in vivo* of the D-isomer of methionine to the L-antipode, or else that either the D- or the L-isomer of methionine is equally available in the synthesis of cysteine.

In 1932 Butz and du Vigneaud (7) observed the formation of homocystine during the decomposition of methionine in strong sulfuric acid. Obviously, homocystine could not have arisen from methionine unless the latter was demethylated and oxidized under these conditions. The possibility of the demethylation of methionine to homocysteine *in vivo* was thus anticipated by the formation *in vitro* of homocystine from methionine, and was strongly supported by the availability of either isomer of homocystine in lieu of cystine in the diet of the rat (8). In 1939 Tarver and Schmidt, by employing methionine-S<sup>35</sup>, demonstrated the incorporation of the sulfur of methionine into that of cystine in the rat (9). It now remained to show the origin of the carbon skeleton of cysteine.

Stetten (10) administered serine-N<sup>15</sup> to rats and recovered a considerable proportion of the N<sup>15</sup> in the tissue cystine. Shemin (11) recovered the N<sup>15</sup> of administered serine-N<sup>15</sup> in the isolated glycine of hippuric acid in the rat, a result suggesting for the first time, so far as we know, the formation of formate from the beta carbon of serine in the course of glycine synthesis from serine. The existence of formate and formaldehyde in animal tissues had been known for many years prior to that. The agitation about "single-carbon units" is only of recent origin, and it is obvious from the literature that it is not the discovery of these single-carbon units that is responsible for the activity, but a realization that an important metabolic net of pathways involving these units exists, one of which has led to



the discovery of the synthesis *de novo* of the methyl group of methionine *in vivo* and *in vitro*.

Several theories have been proposed in regard to the origin of the carbon skeleton of cysteine. Nicolet (12) proposed the formation of  $\text{H}_2\text{S}$  from homocysteine and its addition to aminoacrylic acid. Brand, Block, Kassell, and Cahill (13) suggested the interaction of homocysteine with aminoacrylic acid to yield a thioether (now known as cystathionine) which cleaved to form cysteine. Toennies (14) postulated the interaction of methionine with serine to form a sulfonium compound which split off methanol to give cystathionine. The latter is then cleaved to cysteine. In addition to these theories, two theories were proposed in which the carbon chain of methionine was shortened to yield the three-carbon skeleton of cysteine. One theory proposed demethylation of methionine to homocysteine, followed by an oxidative degradation at the aminocarboxylic acid end of the chain (15). The other theory suggested a shift of the sulfur of homocysteine, followed by omega oxidation and decarboxylation (9).

The last two theories, which propose that the carbon of cysteine originates from the aminobutyric acid moiety of methionine, proved to be untenable by the observation that methionine, labeled with deuterium in the beta and gamma carbons, gave rise in rats to cystine which was practically free of deuterium (15). In addition, thiothreonine, proposed by Tarver and Schmidt (9) as the intermediate in the conversion of the carbon chain of methionine to that of cysteine, proved to be incapable of supporting growth of rats in lieu of cystine (16).

The schematic outline of the theory of Brand, Block, Kassell, and Cahill (13) for the formation of cysteine from methionine is shown in Fig. 2. The suggested scheme took into account the proposal of Nicolet (17) that aminoacrylic acid was the possible source of the carbon skeleton of cysteine, and the report of Kuester and Irion (18) on the isolation of S-( $\alpha$ -carboxyl-aminoethyl)-homocysteine, the structure of which Kuester and Irion deduced on the basis of somewhat incomplete evidence. This thioether has



later been named cystathionine by du Vigneaud and coworkers (19). The experimental evidence for the validity of this theory has been provided by subsequent work in several laboratories.

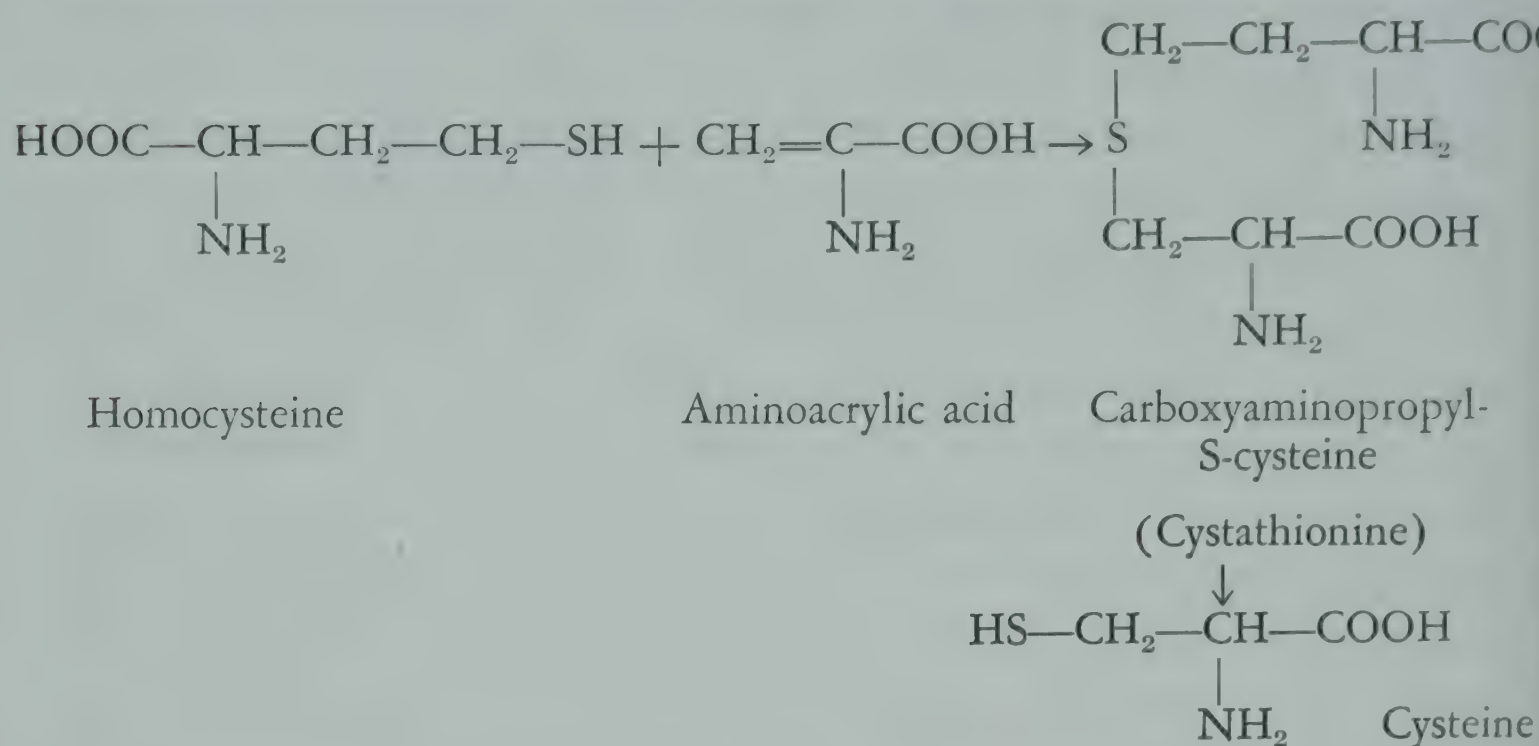


FIG. 2. A scheme for the possible formation of cysteine from homocysteine in vivo. (According to E. Brand, R. J. Block, B. Kassell, and G. F. Cahill, *Proc. Soc. Exptl. Med.*, 35, 501, 1936; based on model experiments of B. H. Nicolet, *Science*, 81, 181, 1935 and on the isolation of "cystathionine" from wool by Küester and Irion, *Z. physiol. Chem.*, 184, 225, 1929).

All four diastereoisomers of cystathionine were synthesized (20) and tested on rats for growth in lieu of either cystine or methionine in the diet. L-Cystathionine supported the growth in lieu of cystine only; L-allo-cystathionine supported the growth in lieu of methionine only if choline was also present in the diet; neither of the D-diastereoisomers of cystathionine would promote growth in lieu of cystine or methionine. It was deduced from these growth experiments that L-cystathionine is cleaved in vivo to cysteine, and L-allo-cystathionine is cleaved to homocysteine. Studies with rat liver preparations confirmed these deductions (21). Cysteine was actually isolated from liver digests which contained L-cystathionine. Along with cysteine,  $\alpha$ -ketobutyric acid was isolated as the hydrazone. Homoserine, proposed as the other cleavage product of L-cystathionine in vitro, apparently was deaminated and reduced to the ketobutyric acid. L-Cystathione-S<sup>35</sup> was also fed to rats and S<sup>35</sup>-

cystine was isolated from their hair (22). In our laboratory, we have recently synthesized L-cystathionine labeled with  $C^{14}$  in carbon-3 of its aminopropionic acid end, and have administered it to rats and chicks. From the hair and feathers respectively, L-cystine-3- $C^{14}$  was isolated. Whole liver homogenates of rats and chicks, incubated with L-cystathionine- $C^{14}$ , yielded cysteine-3- $C^{14}$  (23).

When methionine was incubated with liver preparations, little if any cysteine was formed. However, when homocysteine and serine were incubated in the same preparations, about a 60 per cent yield of the sulfur of L-homocysteine in the form of cysteine was obtained (24). Methionine, labeled with  $S^{34}$  and with  $C^{13}$  in the beta and gamma carbons, was fed to rats, and the isolated cystine contained only the  $S^{34}$  (25), a result once again confirming the theory that the beta and gamma carbons of methionine are not available for the formation of the carbon skeleton of cysteine.

Further evidence for the biological significance of L-cystathionine was obtained by Horowitz (26) in a mutant of *Neurospora* which accumulated the thioether in the medium, and by Genghof (27), who observed that *Tetrahymena geleii* could utilize cystathionine as a precursor of cystine but not of methionine.

Thus, originally an artifact, cystathionine became a biological intermediate in the synthesis of cysteine in vivo. We shall see a little later that two more artifacts were prepared which serve in vivo as precursors of cystine. In addition, a presumed antimetabolite of methionine, ethionine, which apparently is about to cease to be an artifact, as a result of the work of Schlenk (28), also yields its sulfur to cysteine in the rat (29). These remarks should not in any way minimize the importance of cystathionine in cysteine synthesis. Our feeling in the matter is that the whole story of cysteine synthesis and of the metabolic role of cystine-cysteine has not yet been told.

The isolation of  $\alpha$ -ketobutyric acid along with cysteine from an incubation mixture containing homocysteine and serine leaves the question open as to whether the ketobutyric acid was a direct cleavage product of cystathionine or whether it arose from homoserine. The isolation by Dent of  $\alpha$ -aminobutyric acid from the urine of patients



with a low renal threshold for amino acids (30) or from the urine of normal individuals who had ingested methionine (31) is in line with the experiments *in vitro* just described. Neither data, however, provide a real clue as to the direct precursor of either the ketobutyric acid or the aminobutyric acid, or as to whether one originated from the other, although it seems reasonably certain that either acid originated from methionine, possibly via cystathionine.

Subsequent studies by Binkley (32) indicated that the enzyme responsible for the cleavage of L-cystathionine to cysteine is stable at 50° C., whereas the homocysteine-serine condensing enzyme is not. This property was utilized in studies of the two enzyme systems separately. The cystathionine-cleaving portion of the preparation cleaved several thioethers, in addition to L-cystathionine, suggesting either nonspecificity of the enzyme or the crude state of the preparation. Thus, djenkolic acid, which cannot replace cystine in the diet of rats (33), lanthionine, and L-cystathionine were cleaved to cysteine, while L-allo-cystathionine was cleaved to homocysteine. L-Cysteine yielded H<sub>2</sub>S and several S-alkyl-substituted cysteines or homocysteines, including methionine, yielded the corresponding alkyl sulfides (34). It is not clear whether cysteine desulfhydrase, obviously present in the preparation, is a contaminant, or whether the product of the cleavage by "thionase" (thioether-cleaving enzyme) is determined by the nature of the groupings situated on either side of the sulfur of the thioether.

Thionase was later obtained in a state of considerable purity (35) from dog livers. The activity of the enzyme was easily lost in aqueous solutions and it was destroyed by freezing. In saline solution, half of the activity was lost in 3 days at 4° C. The purified preparation retained its ability to form H<sub>2</sub>S from cysteine, however. ATP was not required for the activity of the enzyme (35). Preliminary data on the apparent activation of the dialyzed preparation of thionase by folic acid has also been reported by Binkley (36).

Not until 1950, it seems, was it suspected that pyridoxal phosphate plays a key role in the synthesis of cysteine from homocysteine and serine. Almost ten years before that, however, Cerecedo and Foy



(37) had reported that the onset of pyridoxine deficiency in rats is greatly accelerated by a high content of casein or methionine in the diet—a fact suggesting the involvement of pyridoxine in the metabolism of methionine. In 1950 Braunshtein and Goryachenkova (38) reported that rat liver homogenates of pyridoxine-deficient rats do not form cysteine from homocysteine and serine; that the addition of pyridoxal phosphate to pyridoxine-deficient liver homogenates restores the ability to synthesize cysteine from homocysteine and serine; that hydroxylamine, added to normal liver homogenates, inhibits cysteine formation from homocysteine and serine; and finally, that pyridoxine injected into the pyridoxine-deficient rats restores the ability of liver homogenates to form cysteine. Binkley et al. (39) also reported later that pyridoxal phosphate appears to be the coenzyme for the homocysteine-serine condensing enzyme as well as for thionase, but that, in contrast to Binkley's earlier observation (36), the activity of these enzymes was not affected by treating rats with 4-aminopteroylglutamic acid.

Metzler and Snell (40) obtained a preparation from vitamin-B<sub>6</sub>-deficient cells of a mutant strain of *Escherichia coli* which contained serine dehydrases that required pyridoxal phosphate as coenzyme. The D-serine dehydrase was not activated by adenosine-5-phosphate, while L-serine dehydrase was. Chargaff and Sprinson (41) have suggested that in this reaction the enzyme catalyzes the dehydration of serine to  $\alpha$ -aminoacrylic acid, which is then spontaneously converted to pyruvate and ammonia. Like the suggestion made for the non-enzymatic dehydration of serine by pyridoxal phosphate or pyridoxal (42), Metzler and Snell (40) have proposed that a Schiff base is formed between pyridoxal phosphate and serine, followed by dehydration to a Schiff base of pyridoxal phosphate and  $\alpha$ -aminoacrylic acid. The scheme proposed by Metzler and Snell (40) is illustrated in Fig. 3. Whether this enzymatic reaction also requires a metal ion, like the non-enzymatic reaction, has not yet been determined. The presence of the long conjugated system in the Schiff base of aminoacrylic acid would stabilize the base, and the presence of the double bond would permit the addition of homocysteine across



the double bond to give cystathionine, as Metzler and Snell (40) have suggested.

With these important additional experimental details, the scheme of Brand, Block, Kassell, and Cahill (13) for the formation of cysteine from homocysteine has withstood the test. Several details are still to be added to the main scheme. One of these is the

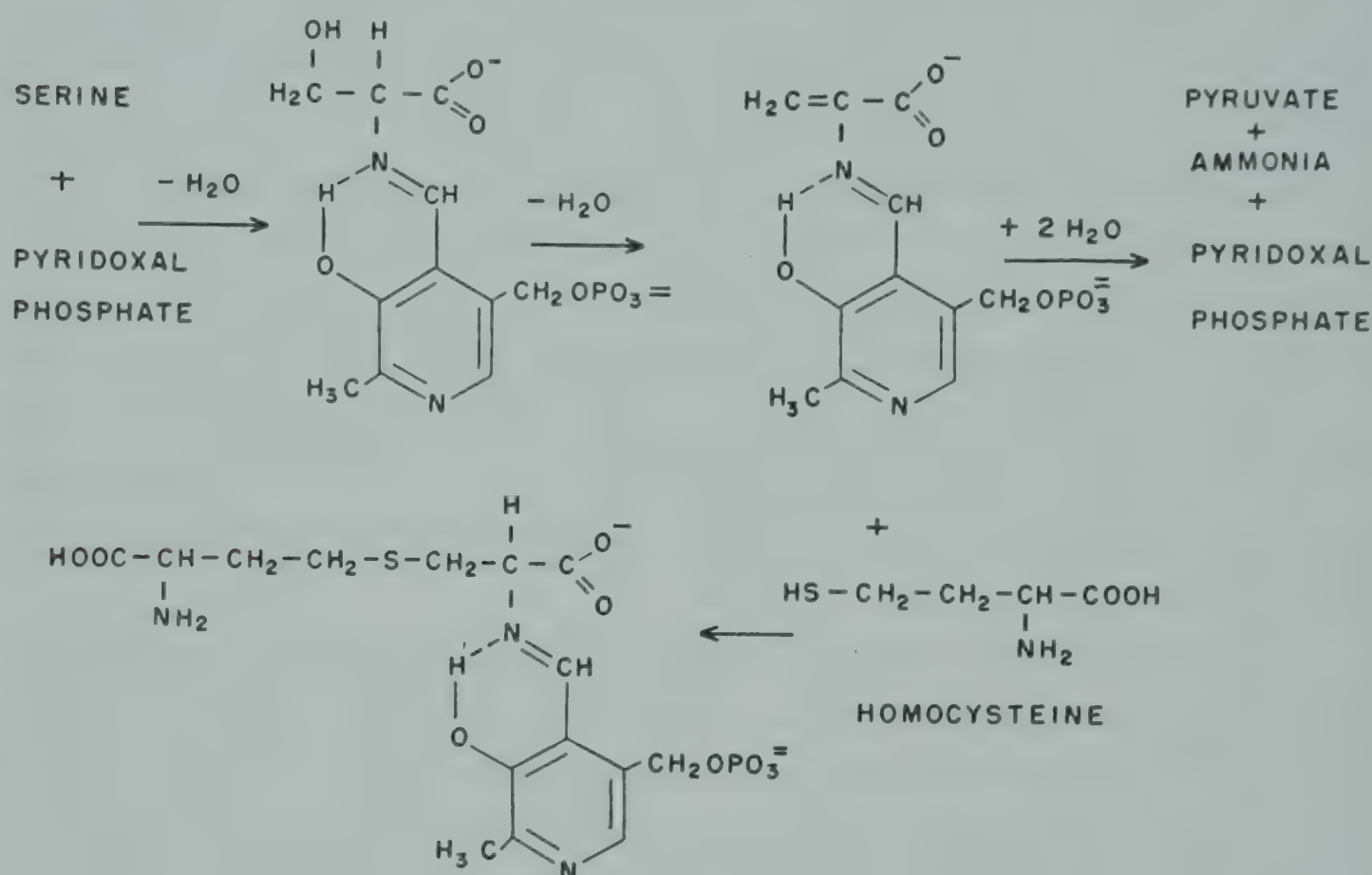


FIG. 3.

metabolic nature of homocysteine that originates from methionine on demethylation. Recent observations by Cantoni (43) have centered attention on adenosylmethionine sulfonium base as the intermediate in the process of demethylation of methionine. If S-adenosyl-homocysteine is the product of demethylation of methionine, one wonders whether adenosyl-homocysteine is the actual participant in the condensation with the Schiff base of aminoacrylic acid, or whether adenosyl-homocysteine cleaves to homocysteine and adenosine prior to the condensation. Purification of the system or systems which produce cystathionine from homocysteine and serine, and further study of the cofactors involved in these systems, are problems awaiting solution. It is of interest to note that rat pancreatic tissue is

apparently a richer source of the enzymatic systems which form cysteine from homocysteine and serine than the liver (44).

It was mentioned earlier that a considerable proportion of dietary methionine can be replaced by cystine, although an absolute minimum requirement for methionine in the diet of animals must be assured. The supplementation of the diet by cystine apparently "spares" the animal the necessity of manufacturing cystine from methionine. Thus, the ability of the animal to synthesize the required cystine and the availability of the necessary precursors and cofactors in this synthesis become of paramount importance. As far as we know, no systematic investigation of the extent of cysteine synthesis in animals in relation to age, species, and variation in dietary composition has been published. The study could employ several approaches simultaneously: nutritional, measurement of the enzymatic activities of systems involved in cysteine formation, and the isotope tracer technique. Such an investigation is important from the standpoint of the supplementation of diets low in methionine with cystine, particularly the diet of human beings. A recent note by Rose et al. (45) calls attention to the fact that a large part of the human race subsists on diets known to be low in methionine. An early observation of Weichselbaum (46) that rats maintained on diets low in cystine but ample in methionine develop liver lesions and stop growing, and that the addition of *methionine* to the diet failed to alleviate the situation, while the addition of *cystine* was effective in that respect, further calls attention to the possibility that prolonged maintenance of animals on diets low in cystine might reveal the inadequacy of the diet and the inability of the animal to overcome that inadequacy, if it is forced to meet it over a prolonged period of time, in spite of the presence of methionine in the diet. The adequacy of the enzymatic systems to convert methionine to cystine in animals must, therefore, be evaluated in terms of need, time, and the available precursors and cofactors involved in the synthesis.

The interconvertibility of glycine and serine and the demonstration of the role of "formate" in that reaction immediately suggested



the possibility that the  $\alpha$ -carbon of glycine and all metabolites which yield "formate" would contribute to the "serine pool" and hence to the carbon skeleton of cysteine. Several such "formate" or "active formaldehyde" sources are known, and the list of compounds which contribute to the "serine pool" and hence to the carbon moiety of cysteine is shown in Table 1.

TABLE 1

COMPOUNDS WHICH CONTRIBUTE TO THE CARBON CHAIN OF CYSTEINE IN VIVO

To 3-carbon of cysteine	To 1,2,3-carbons of cysteine
Methanol	1,2-carbons of glycine
Formate	1,2,3-carbons of serine (L-)
Formaldehyde	alpha-amino-propionic acid
Methyl group carbon of choline, betaine, dimethyl-glycine, sarcosine, methionine	end of L-cystathionine
2-carbon of histidine	1,2-carbon of threonine (L-)

All the compounds listed contribute to the corresponding positions of serine, except L-cystathionine.

Plaut, Bethel, and Lardy (47) noted that in folic-acid-deficient rats the carbon of formate is not incorporated into the  $\alpha$ -carbon of serine. This observation suggested the inference that the deficiency in folic acid would decrease the extent of the "serine pool" and the availability of all "active formaldehyde" donors for serine and cysteine formation. Indeed, the folic acid deficiency in the rat decreased the extent of incorporation of the  $\alpha$ -carbon of glycine into the cysteine and glutamic acid moieties of glutathione (48), and of the carbons of formate, the methyl group carbon of methionine, and of the  $\alpha$ -carbon of glycine into tissue cysteine of the rat (49). Considerable evidence has accumulated to suggest that one or more folic acid derivatives act in vivo as the "carriers" of the "single carbon moiety," which originates from several metabolic sources, to the beta position of serine, to purines, to thymine, and, perhaps, to methionine and other metabolites. The role of the folic acid derivative as a coenzyme in the synthesis of purines focuses attention on the possible indirect effect of folic acid deficiency in whole animals

on various metabolic interconversions in which certain purine nucleotides may be or are involved. This point will be raised once more in connection with the discussion of the effect of folic acid deficiency on the transfer of the methyl group of methionine and choline to various acceptors.

TABLE 2

## COMPOUNDS WHICH CONTRIBUTE THEIR SULFUR TO CYSTEINE IN VIVO

Methionine	$\text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CH-COOH}$ <div style="text-align: center; margin-left: 150px;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div>	(L or D)
All compounds that give rise to methionine		
Homocystine	$\text{HOOC-CH-CH}_2\text{-CH}_2\text{-S-S-CH}_2\text{-CH}_2\text{-CH-COOH}$ <div style="display: flex; justify-content: space-around; margin-top: -10px;"> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> </div>	(L or D)
Homocysteine or its thiolactone	$\text{HOOC-CH-CH}_2\text{-CH}_2\text{-SH}$ <div style="text-align: center; margin-top: -10px;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div>	
L-cystathionine or L-allo-cystathionine	$\text{HOOC-CH-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH-COOH}$ <div style="display: flex; justify-content: space-around; margin-top: -10px;"> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> </div>	
Lanthionine	$\text{HOOC-CH-CH}_2\text{-S-CH}_2\text{-CH-COOH}$ <div style="display: flex; justify-content: space-around; margin-top: -10px;"> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> </div>	(L)
Homolanthionine	$\text{HOOC-CH-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-CH-COOH}$ <div style="display: flex; justify-content: space-around; margin-top: -10px;"> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> <div style="text-align: center; width: 40%;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div> </div>	(L)
Ethionine	$\text{HOOC-CH-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_3$ <div style="text-align: center; margin-top: -10px;"> <math>\downarrow</math>  <math>\text{NH}_2</math> </div>	(L or D)
$\text{Na}_2\text{SO}_4$	Bacteria of rumen of goats, sheep, cows; plants; rabbits, chickens	

Table 2 lists compounds which have been shown to contribute their sulfur to cysteine in intact animals, or in vitro, or in bacteria. L-Lanthionine, an artifact which was isolated from alkali-treated wool, promotes the growth of rats in lieu of cystine (50), and the compound is readily cleaved by liver preparations to cysteine (34). Homolanthionine, another artifact, which has not yet been found in nature, also promotes the growth of rats in lieu of cystine, but not in lieu of methionine (51). Its sulfur, labeled with  $S^{35}$ , was



recovered in cystine or rat tissues (52). Homolanthionine could not be cleaved to homocysteine in rat liver, kidney, spleen, intestine,

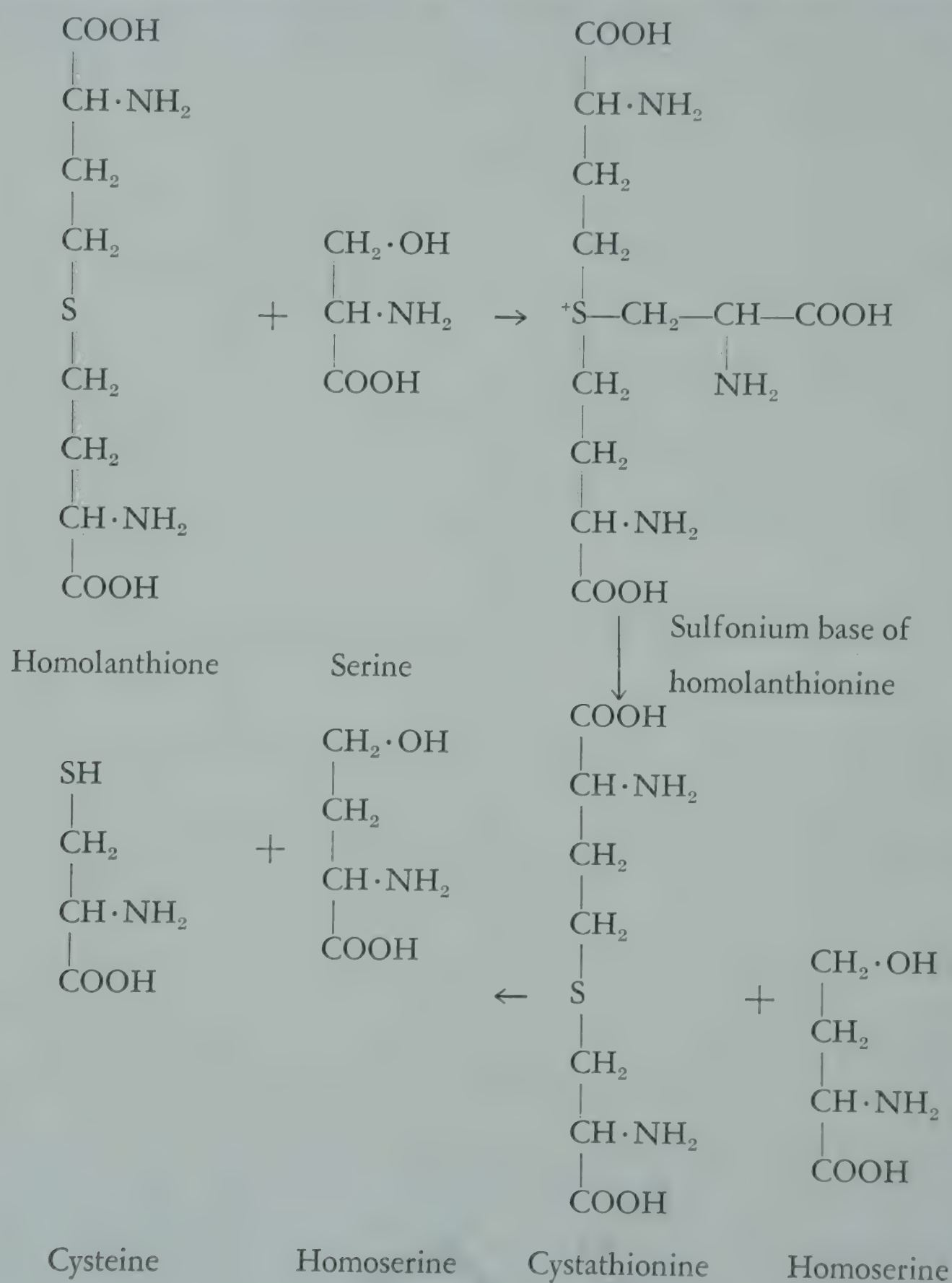


FIG. 4. A scheme for the formation of cysteine from homolanthionine.

or muscle preparations (53). A tentative scheme which illustrates the possible pathway of cysteine formation from homolanthionine

(51) is shown in Fig. 4. It is analogous to the scheme proposed by Toennies (14) for the formation of cystathionine from methionine and serine through the intermediate formation of a methylcystathionine sulfonium base. No further information is available in regard to the manner in which homolanthionine yields cysteine in the rat, except that only the L-isomer of the compound is active in this respect (53).

The carbon of the ethyl group of ethionine, labeled with  $C^{14}$ , was found to be transferred to choline and creatine in rats, and its sulfur, labeled with  $S^{35}$ , was recovered in tissue cysteine (29). Apparently, the ethyl group of ethionine was removed in vivo, probably by mechanisms analogous to those involved in the transfer of the methyl group of methionine. The possible formation of "active ethionine," adenosylethionine, through the interaction with ATP was thus indicated, by analogy with the interaction of methionine with ATP (54). The recent isolation of thioethyladenosine from yeast which had been grown in the presence of ethionine- $C_2H_5-C^{14}$  (28), strongly supports this inference. The utilization of the sulfur of ethionine for cysteine formation has taken place probably through the intermediate formation of homocysteine from ethionine. We recently administered ethionine- $C_2H_5-C^{14}$  and ethionine-2- $C^{14}$  to rats, and from the tissue proteins isolated methionine. The concentration of  $C^{14}$  in methionine isolated after the administration of ethionine-2- $C^{14}$  was almost 10-fold greater than after the administration of ethionine- $C_2H_5-C^{14}$ . Had ethionine been incorporated into tissue proteins without any preliminary deethylation followed by methylation, the concentration of the  $C^{14}$  in tissue methionine after the administration of ethionine, labeled in the ethyl group or in the  $\alpha$ -carbon, would have been the same (55).

Administration of  $Na_2SO_4-S^{35}$  to goats, sheep, and cows resulted in considerable incorporation of the  $S^{35}$  into the methionine and cystine of the milk (56), probably to a large extent through the mediation of the bacteria of the rumen. However, rabbits which were denied access to their feces also incorporated the  $S^{35}$  of  $Na_2SO_4$  into tissue cystine (57). Hens fed  $Na_2SO_4-S^{35}$  laid eggs from which





cystine-S<sup>35</sup> could be isolated (58). The existence in bacteria and in higher organisms of the sequence of metabolic reactions shown in Fig. 5 is not unreasonable, although it is admittedly speculative and probably inadequate.

The reduction of sulfate sulfur to hydrogen sulfide (Reaction 1) is well known. Reaction 2 represents the formation of aminoacrylic acid from serine by the action of serine dehydrase, as discussed earlier. Reaction 3 represents the reverse reaction of cysteine desulfhydrase (59). Reaction 4 shows the possible formation of homoserine from aminobutyric acid or from formaldehyde and pyruvate. It has been demonstrated by Hift and Mahler (60) that an enzyme exists in beef liver which condenses formaldehyde with pyruvate to  $\alpha$ -keto- $\gamma$ -hydroxybutyric acid. The amination of the keto acid to homoserine would be the reverse of the deamination of homoserine (61). Reaction 5 represents the reverse reaction of the cleavage of cystathionine, and Reactions 6 and 7 are the reverse reactions for the synthesis of cysteine from methionine via cystathionine.

### SYNTHESIS OF METHIONINE

It is clear by now that the methyl group of methionine can originate in vivo or in vitro by means of a transfer to homocysteine of the methyl group which is attached to an onium pole in compounds such as betaine, dimethylthetin, or dimethylpropiothetin, or that the methyl group of methionine can be synthesized de novo from the one-carbon units derived from numerous metabolites. The direct transfer of the methyl group to or from methionine in vivo has been designated by du Vigneaud and his coworkers as transmethylation. It has been pointed out by du Vigneaud that there is no reason to suppose that the methyl group of methionine which has arisen by synthesis de novo is metabolically distinct from the methyl group of methionine which has arisen via the process of transmethylation. Therefore, the methyl group of methionine, regardless of its origin, once having been transferred to various acceptors to yield choline, creatine, N'-methyl-nicotinamide, etc., is also metabolically the same. It must be pointed out, however, that the



mechanisms which are involved in the transfer reaction to and from methionine and those which are concerned with the synthesis *de novo* of the methyl group of methionine are distinct biological systems, involving different substrates, cofactors, and enzymes.

The demonstration that rats and other species of animals will grow on diets which contain homocystine, but which are free of the preformed methyl group donors, only if vitamin B<sub>12</sub> and folic acid are also present in the diet, was suggestive of the possible involvement of either or both vitamins, either in the synthesis *de novo* of the methyl group of methionine, or in the transmethylation reactions, or in both processes. Suggestions were also made that vitamin B<sub>12</sub> is concerned only with the reduction of the disulfide bond of homocystine to the sulfhydryl of homocysteine (62) or in the reduction of certain sulfhydryl enzymes (63), and not with the formation of the methyl group *de novo*. The role of folic acid was attributed to its activity as a "carrier" of the one-carbon moiety in a variety of synthetic reactions, including the synthesis of the methyl group of methionine. Thus, the effectiveness of vitamin B<sub>12</sub> and of folic acid in the apparent synthesis of methionine from homocystine in intact animals, as indicated by their growth, could not be obviously ascribed to any one of the implicated possibilities without some detailed further study.

The synthesis *de novo* of the methyl groups of choline, creatine, methionine, and thymine from a variety of one-carbon precursors has been established by the isotopic tracer approach in intact animals and *in vitro*. Cantoni (1) expressed the opinion that "the results from such an approach cannot be taken to imply that a new methyl group has been actually formed in the course of the synthesis of choline and creatine." In the opinion of my coworkers and myself, the results from such studies demonstrate that the labeled carbon of the administered precursor found its way *in vitro* and *in vivo* into the methyl groups of the isolated metabolites. Since the labeled carbon of most of the precursors was not that of an intact methyl group, and since none of such precursors is known as a "methyl donor," the methyl group of the isolated metabolites must have been



synthesized de novo and then perhaps, transferred to some of the metabolites via the mechanisms of transmethylation. We also do not share the opinion of Cantoni (1) that "no indication is supplied by this approach as to which is the first compound in which  $C_1$ -precursor appears as a methyl group." Perhaps it would be fairer to state that no approach to date has supplied this information, simply because the problem is young, the means and abilities are limited, and the work is in progress, and not because the isotopic tracer approach, used judiciously, is incapable of furnishing a reasonably good answer to this problem.

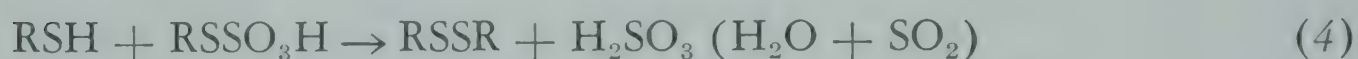
In fact, it was by the tracer approach that the group at Western Reserve University arrived at the opinion, which we shared for some time, that the methyl group of methionine is formed by condensation of the  $C_1$ -fragment with homocysteine and reduction, and not secondarily by transmethylation from choline or betaine (64). The same approach was used by the group at Western Reserve University to implicate folic acid in the utilization of formate in the synthesis of the methyl groups of methionine and choline (65).

Before proceeding with the presentation of data on the synthesis of methionine, it appears apropos to discuss briefly the methods employed in the isolation of the methyl group of methionine, or of methionine itself, from biological materials. In our laboratory, we have hesitated to use the commonly employed procedure of Baernstein (66), in which the methionine present in biological materials is subjected to digestion with hot HI. The liberated methyl iodide is trapped, usually in trimethylamine, then assayed as the resulting tetramethylammonium iodide. The method appears to be an excellent one for the estimation of methionine in *pure proteins*. We were not at all certain that the biological materials that we worked with were pure proteins, since they were obtained from animal sources after dosing the animals with radioactive methionine precursors. We had to be certain that the methyl iodide which arises in the course of digestion with hot HI originated exclusively from the methyl group of methionine. We therefore awaited the completion of the development of a method for the isolation of methio-



nine from crude animal sources in pure form, with good yields, requiring a relatively small amount of starting material, and so relatively uncomplicated as to require minimum time and labor. Such a method was finally developed at our Institute by Dr. T. F. Lavine and Mr. N. F. Floyd, whom I wish to thank for making the procedure available to us long before its publication. Time permits only a brief description of the principles involved in the method, and of certain criteria which we applied to the procedure to insure its applicability to our purpose.

Butz and du Vigneaud (7) described the preparation of homocystine from methionine by boiling the latter in strong sulfuric acid.



or

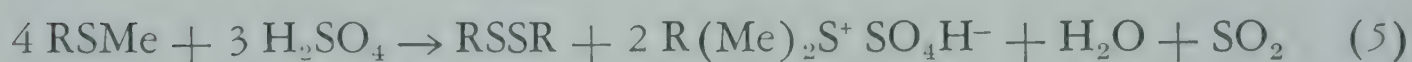


FIG. 6. Decomposition of methionine in sulfuric acid

(According to T. F. Lavine and N. F. Floyd, *J. Biol. Chem.* 207, 97, 1954).

Symbols: RSM<sub>e</sub>, methionine; Me, methyl group.

Lavine and Floyd (67) studied the details of this decomposition, and the reactions involved are shown in Fig. 6. The important observation made by Lavine and Floyd was that methyl methionine sulfonium was also formed in the course of the decomposition of methionine. The methylation reaction is analogous to the methylation of methionine with methyl halides to give methyl methionine sulfonium, observed by Toennies and Kolb (68). Digestion of methionine together with aliphatic or aromatic alcohols in sulfuric acid yielded, with a few exceptions, the corresponding sulfonium bases (69). Digestion of methionine with methanol in sulfuric acid gave methyl methionine sulfonium in quantitative amounts, which was isolated as the bromide, and characterized. The reaction is shown in Fig. 7.

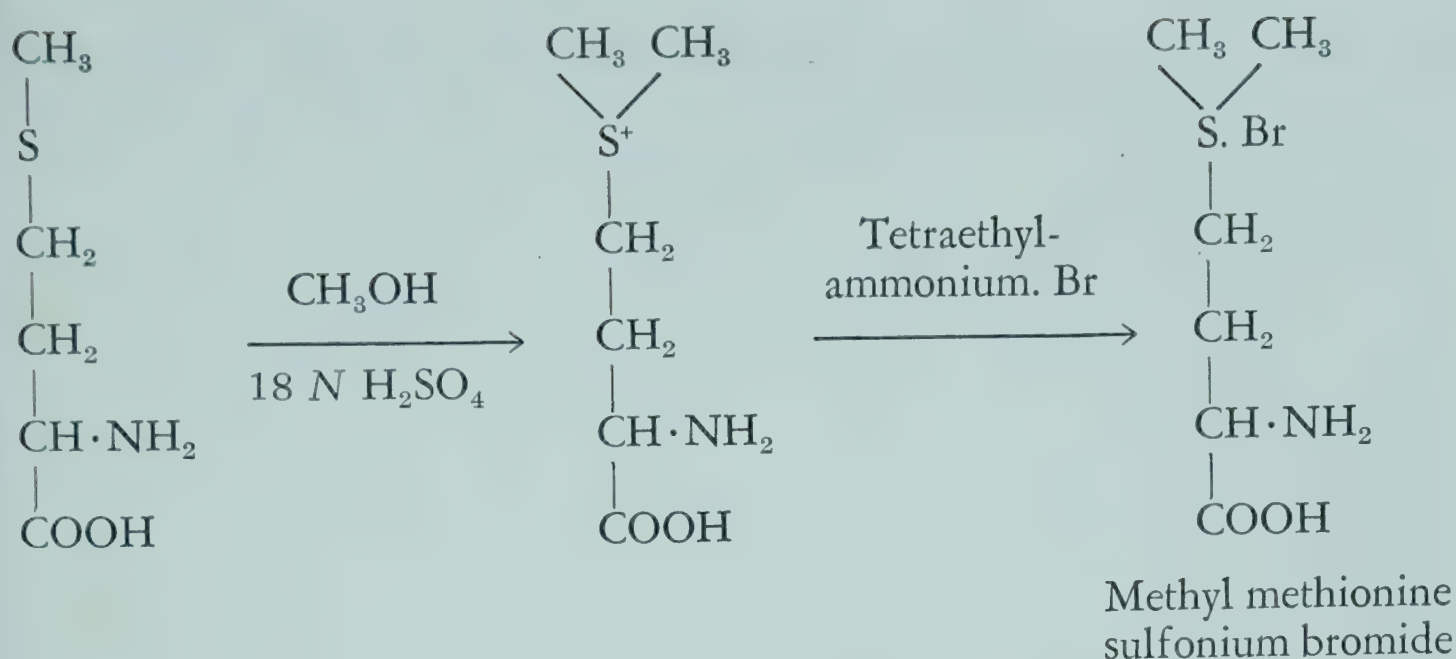


FIG. 7. Formation of methyl methionine sulfonium base from methionine and methanol in sulfuric acid.

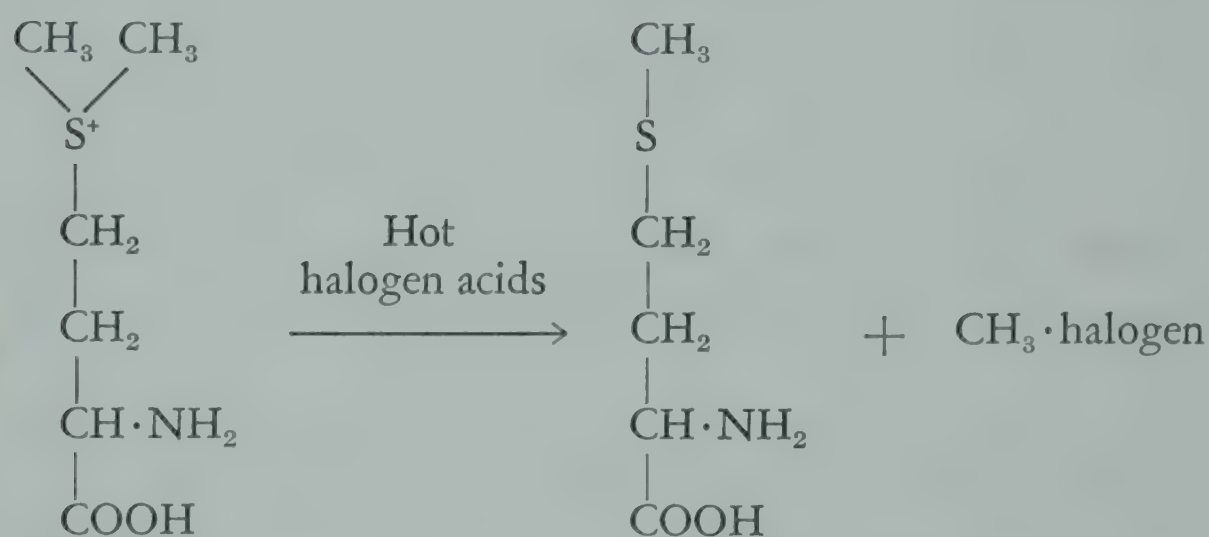
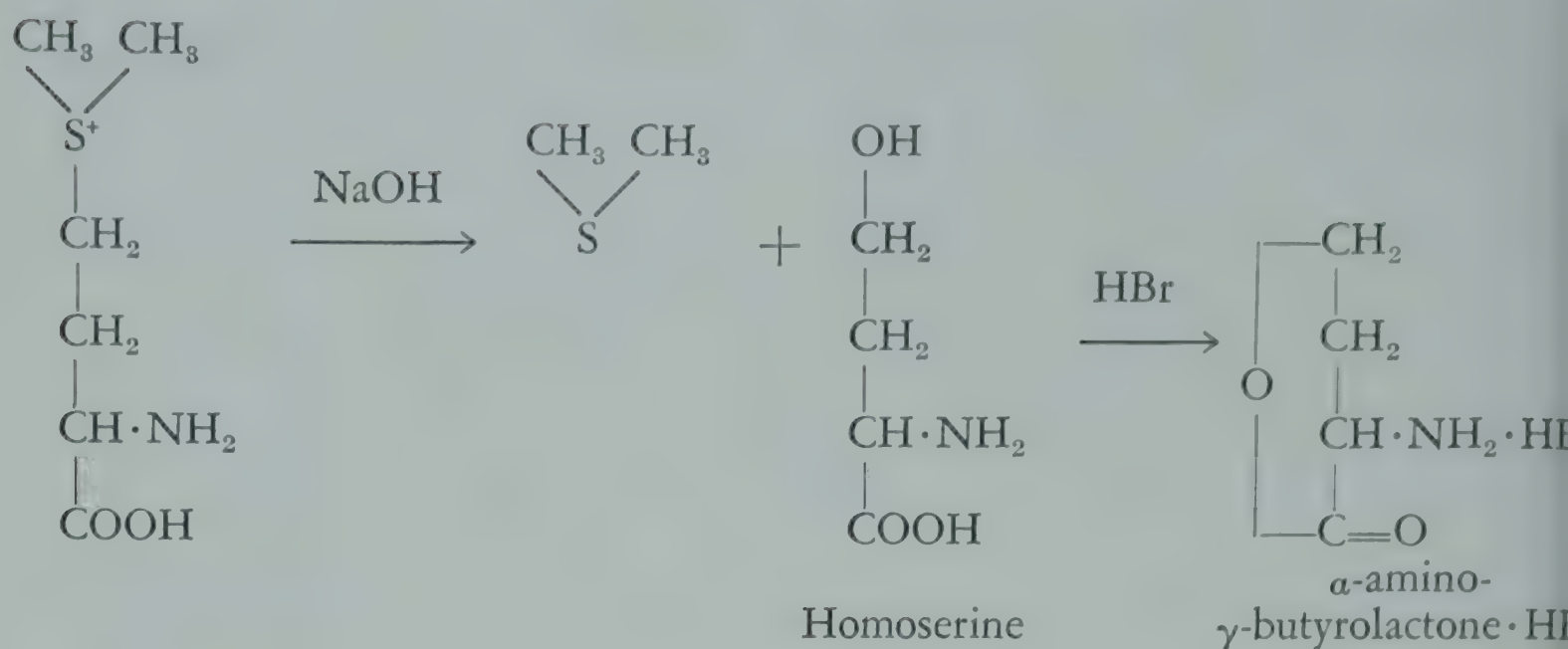
(According to T. F. Lavine et al., *J. Biol. Chem.* 207, 107, 1954).

The compound is easily degraded in either alkali or halogen acids with heat, but not in 16 to 18 N H<sub>2</sub>SO<sub>4</sub>, according to reactions shown in Fig. 8.

These properties of the methyl methionine sulfonium salt permit the location of the labeled atoms of methionine at the strategic positions in the molecule. Of some interest is the degradation of methyl methionine sulfonium in hot HI, with the liberation of methyl iodide. Thus, the presence of aliphatic alcohols, particularly methanol, or of methyl sulfonium compounds in biological materials, would exclude the use of the HI procedure for the isolation of the methyl group of methionine, unless special precautions are taken against the potential methyl contributors.

The absence of any decomposition of the methyl methionine sulfonium salt in hot 16 to 18 N H<sub>2</sub>SO<sub>4</sub> was recorded by Lavine and Floyd (69). To be certain that no exchange of the methyl group of methionine or of methyl methionine sulfonium takes place in the presence of an excess of methanol in a sulfuric acid digestion mixture, we carried out some experiments in which methionine-CH<sub>3</sub>-C<sup>14</sup> or methyl methionine sulfonium-CH<sub>3</sub>-C<sup>14</sup> was digested in 16 to 18 N H<sub>2</sub>SO<sub>4</sub> with varying amounts of methanol. The results are shown in Table 3. No apparent exchange of the methyl group of either





7N HI > 6 N HCl > 6 N HBr > 16 to 18 N H<sub>2</sub>SO<sub>4</sub>, no decomposition being detected in 16 to 18 N H<sub>2</sub>SO<sub>4</sub>.

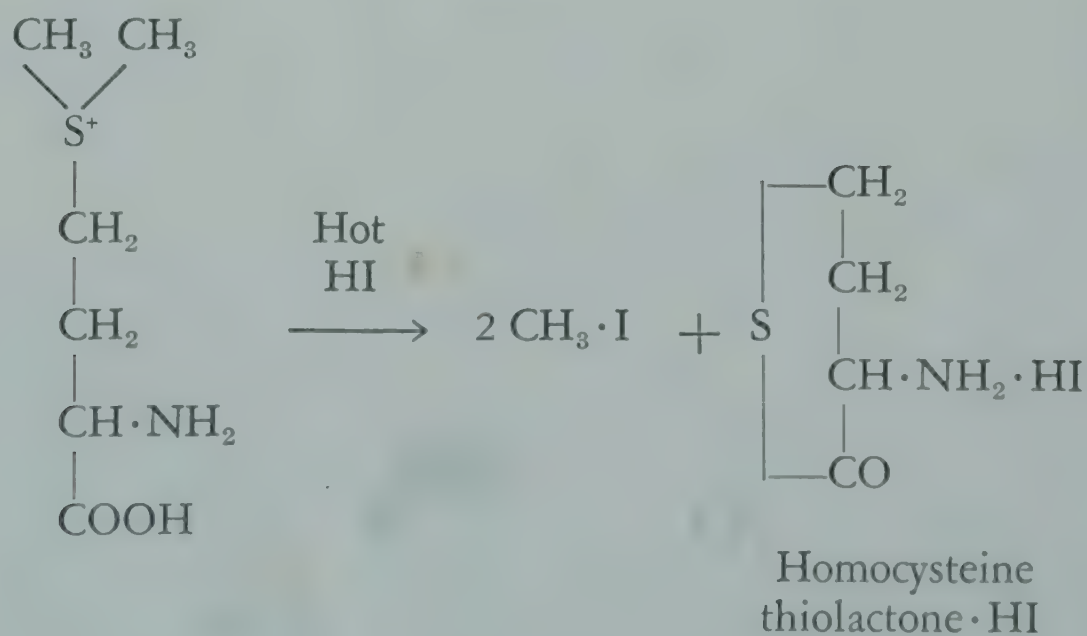


FIG. 8. Degradation of the methyl methionine sulfonium base.  
(According to Lavine et al., *J. Biol. Chem.* 207, 107, 1954).

compound with methanol has taken place under the conditions of isolation of methionine as the methyl methionine sulfonium salt.

TABLE 3

EFFECT OF EXCESS METHANOL ON THE FORMATION OF METHYL METHIONINE SULFONIUM FROM METHIONINE-CH<sub>3</sub>-C<sup>14</sup>, METHIONINE-2-C<sup>14</sup>, OR METHYL METHIONINE SULFONIUM-CH<sub>3</sub>-C<sup>14</sup> IN SULFURIC ACID

	Total activity, c. p. m.	Total activity of methyl methionine sulfonium. Br Equivalents of methanol used			
		1	2	4	8
Methionine-CH <sub>3</sub> -C <sup>14</sup>	14,500	14,560	14,455	14,600	14,530
Methionine-2-C <sup>14</sup>	7,500	7,200	7,480	7,500	7,420
Me-methionine sulfonium-CH <sub>3</sub> -C <sup>14</sup>	6,000	5,970	5,800	5,930	5,950

The compounds were boiled in 16-18 N H<sub>2</sub>SO<sub>4</sub> in the presence of methanol for 30 minutes and the bromide salt of methyl methionine sulfonium was isolated according to the method of Lavine and Floyd (*J. Biol. Chem.*, 207, 107, 1954).

SYSTEM

SOLVENT: TERT. BUTANOL — 70  
FORMIC ACID — 15  
H<sub>2</sub>O — 15

PAPER WHATMAN #1

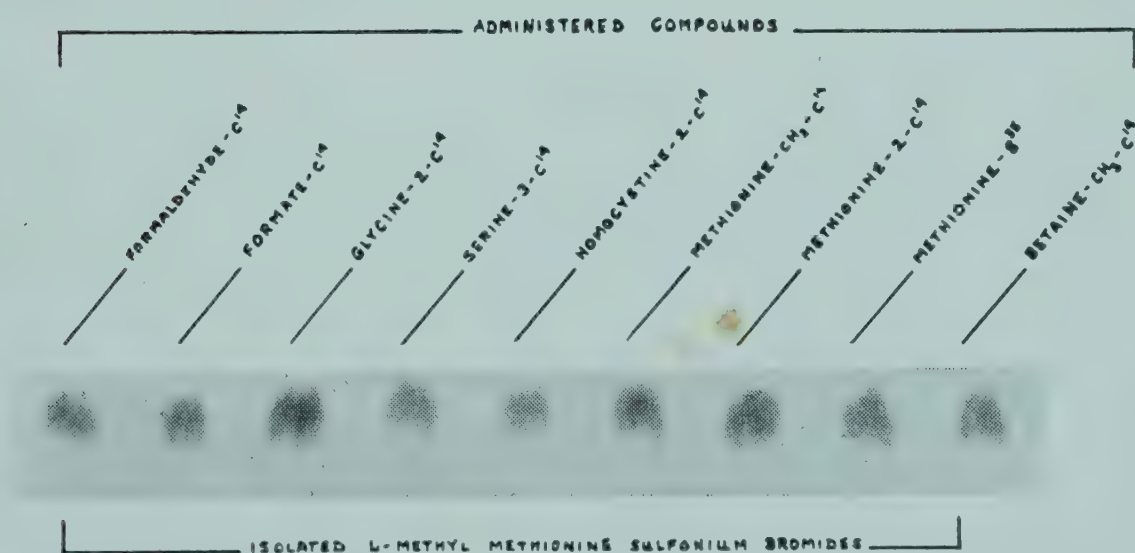


FIG. 9. Chromatogram of samples of methyl methionine sulfonium bromide which were isolated from rat carcasses after dosing the animals with various radioactive precursors of methionine.



Finally, to be sure that the methyl methionine sulfonium salt which we isolated from the carcasses of rats, chicks, and mice contained no undesirable impurity, we subjected the isolated materials to chromatographic analysis, employing several solvent systems. A typical chromatogram on 9 different samples of methyl methionine sulfonium bromides which were isolated from the entire carcasses of rats, after dosing the animals with 9 different radioisotopic precursors of methionine, is shown in Fig. 9. All the data from our laboratory on methionine synthesis were obtained by the procedure of Floyd and Lavine (70), with minor modifications suitable for our specific purpose.

#### TRANSMETHYLATION REACTIONS

Table 4 lists compounds which more or less directly transfer a methyl group to methionine *in vivo* and *in vitro*. In the process of transfer of the methyl group from betaine or the thetins to methionine, no ATP was required. The absence of a requirement for ATP in the transfer reaction is tentatively explainable by the onium structure of these compounds, which is presumably "energy-rich," and does not require further "activation" by ATP. The energetics of the reaction involved in the transfer of the methyl group of betaine to methionine has been considered by Cantoni (1), who employed dimethylthetin as a model compound to show the possible steps involved in the mechanisms of the transfer.

Methyl methionine sulfonium is a thetin. It is a naturally occurring compound (71), and it, as well as methionine sulfoxide, was shown to support the growth of rats (72) and certain strains of bacteria (71). We prepared methyl methionine sulfonium and methionine sulfoxide, labeled with  $C^{14}$  in the methyl group or in the  $\alpha$ -carbon, and studied their metabolism in rats. The availability of either compound for the growth of rats was also determined by employing diets consisting of a mixture of amino acids, in which either compound was the sole source of sulfur-amino acids.

Table 5 summarizes the data obtained in tracer studies. Either the methyl group or the  $\alpha$ -carbon of methionine sulfoxide was well

TABLE 4

COMPOUNDS WHICH MORE OR LESS DIRECTLY TRANSFER A METHYL GROUP TO METHIONINE

Betaine-CH <sub>3</sub> -C <sup>14</sup>	HOOC-CH <sub>2</sub> -N≡(CH <sub>3</sub> ) <sub>3</sub>
Methionine sulfoxide-CH <sub>3</sub> -C <sup>14</sup>	$\begin{array}{c} \text{HOOC}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_3 \\   \qquad \qquad \qquad    \\ \text{NH}_2 \qquad \qquad \text{O} \end{array}$
Methylthetin	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HOOC}-\text{CH}_2-\text{S} \\   \\ + \\   \\ \text{CH}_3 \end{array}$
Methylpropiothetin	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HOOC}-\text{CH}_2-\text{CH}_2-\text{S} \\   \\ + \\   \\ \text{CH}_3 \end{array}$
Methionine methylsulfonium-CH <sub>3</sub> -C <sup>14</sup>	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HOOC}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{S} \\   \qquad \qquad \qquad   \\ \text{NH}_2 \qquad \qquad \qquad + \\ \qquad \qquad \qquad   \\ \qquad \qquad \qquad \text{CH}_3 \end{array}$
Methionine ethylsulfonium-CH <sub>3</sub> -C <sup>14</sup>	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HOOC}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{S} \\   \qquad \qquad \qquad   \\ \text{NH}_2 \qquad \qquad \qquad + \\ \qquad \qquad \qquad   \\ \qquad \qquad \qquad \text{CH}_2-\text{CH}_3 \end{array}$

TABLE 5

UTILIZATION OF METHIONINE SULFOXIDE AND OF METHYL METHIONINE SULFONIUM FOR THE SYNTHESIS OF METHIONINE, CHOLINE, CREATINE, AND SERINE IN THE RAT

Isotope administered *	Methionine	S.S.A. × 100		
		Choline	Creatine	Serine
Methionine-CH <sub>3</sub> -C <sup>14</sup>	10.3	32.0	5.0	0.6
Methionine-2-C <sup>14</sup>	20.1			
Methionine sulfoxide-CH <sub>3</sub> -C <sup>14</sup>	9.1	20.1	5.8	
Methionine sulfoxide-2-C <sup>14</sup>	16.0			
Methylmethioninesulfonium-CH <sub>3</sub> -C <sup>14</sup>	3.1	38.2	6.6	0
Methylmethioninesulfonium-2-C <sup>14</sup>	8.9			

\* Adult female rats were maintained on a diet containing 8% casein, 0.4% threonine, 0.5% cystine, and 1% glycine. All animals were sacrificed 20 hours after the intraperitoneal injection of the isotopes in a single dose. The metabolites were isolated from the entire carcasses.



utilized by the rat. The efficiency of utilization was about 80 to 90 per cent of that shown by similarly labeled methionine, as judged by the extent of incorporation of the labeled carbons into tissue methionine, choline, and creatine. Rats grew well on diets containing methionine sulfoxide, comparable in rate to the growth obtained on methionine. These results confirmed the earlier data obtained with methionine sulfoxide fed together with diets containing arachin as the protein source (73). The rapid and efficient utilization of methionine sulfoxide, injected intraperitoneally, by the rat, suggests the existence in that animal of systems which probably reduce methionine sulfoxide to methionine. Other pathways, leading to the eventual synthesis of methionine, choline, creatine, and serine from the sulfoxide are not, of course, excluded.

The methyl group of methyl methionine sulfonium was utilized well for the synthesis of either choline or creatine, but very little, if any, of the  $C^{14}$  of the methyl group of methyl methionine sulfonium was located in tissue serine, in contrast to the results obtained with the methyl-labeled methionine. Neither the methyl group nor the  $\alpha$ -carbon of methyl methionine sulfonium was utilized for methionine synthesis as well as were those of either methionine or methionine sulfoxide, however. Growth studies on rats ingesting methyl methionine sulfonium as the sole source of sulfur-amino acids revealed poor growth compared to that obtained by feeding methionine under similar conditions. Previous studies showed that rats grow equally well on arachin diets which contained either methionine or methyl methionine sulfonium (72). Arachin is low in methionine but ample in cystine. It occurred to us that the growth of rats fed methyl methionine sulfonium on arachin diets was a result of a combined effect of dietary cystine and of whatever amounts of methionine were elaborated in the rat from the methyl methionine sulfonium fed. Indeed, the addition of cystine to our amino acid diet, which contained methyl methionine sulfonium, produced an immediate acceleration of growth, equal to or better than that obtained on a similar diet containing methionine (74).

It appears, therefore, that the metabolic pathway, or pathways,

of methyl methionine sulfonium in the rat is not exclusively a simple demethylation to methionine. The efficient transfer of the methyl group of methyl methionine sulfonium to choline and to creatine further suggests that the methyl group may not be exclusively transferred via methionine as intermediate. Further studies are needed to elucidate this possibility, as well as that of the transfer of the methyl group from other S-onium compounds to choline, creatine, etc., without the intermediate formation of methionine. We are aware that the methyl group of betaine could not be transferred in vitro to creatine in the absence of homocysteine in the medium (75).

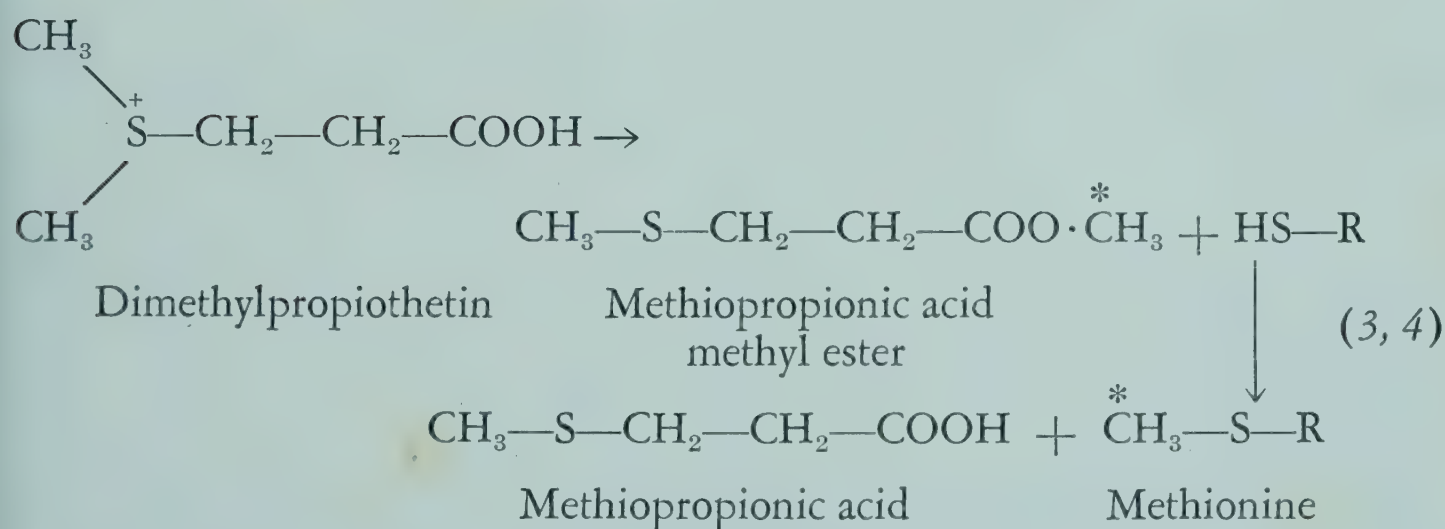
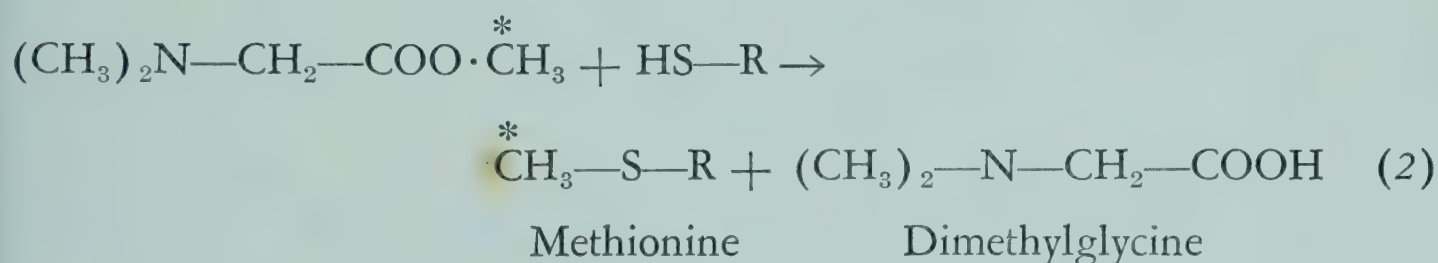
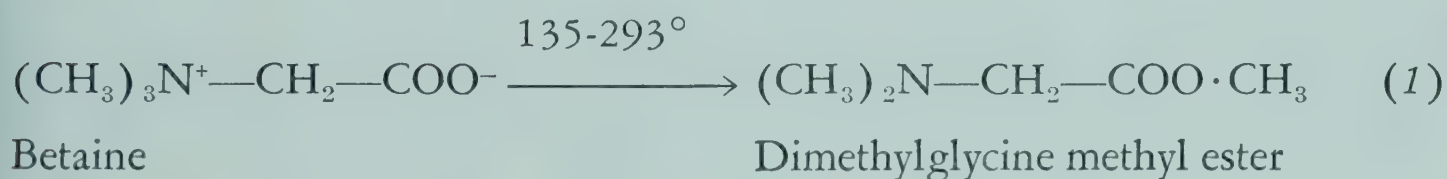


FIG. 10. Postulated reaction sequences whereby a methyl group from betaine or dimethylpropiothetin may be transferred to methionine via the respective methyl esters.

(Reaction 1 according to Willstätter, *Ber. deut. chem. Ges.*, 35, 584, 1902).

At this point I would like to present a series of purely speculative reactions in the course of which the methyl group of the thetins and betaine could be transferred to methionine. In 1902 Willstätter observed the formation of the dimethylglycine methyl ester from



betaine upon heating the latter at 135 to 293° C. (76). The fact that during the biological interaction of betaine or the thetins with homocysteine only one methyl group is transferred to methionine, leaving as the other products dimethylglycine (77) and the corresponding methioacids, suggests the possibility of the biological formation of the corresponding methyl esters from betaine and the thetins. The proposed reactions are shown in Fig. 10. Plans are under way to test these suggestions experimentally in our laboratory.

#### VITAMIN B<sub>12</sub> AND FOLIC ACID IN TRANSMETHYLATION REACTIONS

Previous data from our laboratory have shown that vitamin B<sub>12</sub>-deficiency in rats (78) or chicks (79) had no apparent inhibitory effect on the extent of transfer of the methyl group of methionine to either choline or creatine. Further studies in our laboratory have indicated that vitamin B<sub>12</sub>-deficiency in rats (80) or chicks (81) also

TABLE 6

EFFECT OF DEFICIENCY IN VITAMIN B<sub>12</sub> IN RATS ON THE UTILIZATION OF THE METHYL GROUPS OF CHOLINE AND BETAINES

Isotope administered *	S.S.A. × 100							
	Normal				Vitamin B <sub>12</sub> -deficient			
	Methio- nine	Trime- thyl- amine	Crea- tine	Serine	Methio- nine	Trime- thyl- amine	Crea- tine	Serine
Choline-CH <sub>3</sub> -C <sup>14</sup>	0.22	89.1	0.26	0.50	0.23	98.0	0.3	0.4
Betaine-CH <sub>3</sub> -C <sup>14</sup>	2.13	19.0	3.00	2.70	2.43 *	24.3	2.8	2.6
Methionine-CH <sub>3</sub> -C <sup>14</sup>	10.80	38.0	5.20	0.64	10.50	44.3	5.5	0.5

\* Female rats were maintained on a diet containing 8% casein, 0.4% threonine, 0.5% cystine, and 1% glycine. The "normal diet" contained vitamin B<sub>12</sub>. All animals were sacrificed 20 hours after the intraperitoneal injection of a single dose of the isotopes. The metabolites were isolated from the entire carcasses.

had no apparent inhibitory effect on the transfer of the methyl groups of either choline or betaine to methionine. The data are summarized in Table 6. Of some interest is the relatively poor incorporation of the methyl group of choline into the methyl groups of methionine and creatine, under our experimental conditions. The

efficiency of betaine was nearly 10-fold the efficiency of choline. The possible significance and interpretation of this observation will be discussed a little later.

Earlier reports from our laboratory (78) indicated an inhibitory effect of folic acid deficiency in rats on the transfer of the methyl group of methionine to either choline or creatine. The data on the effect of folic acid deficiency in rats on the transfer reactions to and from methionine are summarized in Table 7. The extent of

TABLE 7

EFFECT OF FOLIC ACID DEFICIENCY IN RATS ON THE UTILIZATION OF THE METHYL GROUPS OF CHOLINE AND BETAINE

Isotope administered *	S.S.A. $\times$ 100							
	Normal				Folic-acid-deficient			
	Methio- nine	Trime- thyl- amine	Crea- tine	Serine	Methio- nine	Trime- thyl- amine	Crea- tine	Serine
Choline-CH <sub>3</sub> -C <sup>14</sup>	0.22	89.1	0.26	0.50	0.09	85.70	0.13	0.09
Betaine-CH <sub>3</sub> -C <sup>14</sup>	2.13	19.0	3.00	2.70	2.80	11.00	1.20	1.47
Methionine-CH <sub>3</sub> -C <sup>14</sup>	10.25	31.00	5.00	0.64	12.90	13.00	1.10	0.18

\* Female rats were maintained on a diet containing 8% casein, 0.4% threonine, 0.5% cystine, and 1% glycine. The "normal diet" contained folic acid. All animals were sacrificed 20 hours after the intraperitoneal injection of a single dose of the isotopes. The metabolites were isolated from the entire carcasses.

incorporation of the C<sup>14</sup> of the methyl group of choline into methionine, creatine, and serine was decreased in this deficiency. On the other hand, the extent of synthesis of methionine from betaine was not. As stated previously, the extent of transfer of the methyl group of methionine to either choline or creatine was depressed by folic acid deficiency. The lowered incorporation of the methyl groups of betaine into choline in the folic-acid-deficient rat, therefore, could have been a result of the inhibition by the deficiency of the transfer of the methyl group of the synthesized methionine to choline and creatine. This is also reflected in the higher concentration of the C<sup>14</sup> in methionine after the administration of the labeled betaine or methionine to folic-acid-deficient rats. Lowered incorporation of the C<sup>14</sup> of labeled methionine, choline, or betaine into the tissue



serine in the folic-acid-deficient rat is possibly a reflection of reduced oxidation of the methyl groups to  $C_1$ -units and of subsequent utilization of these units for serine formation. Lowered oxidation of formate to respiratory  $CO_2$  and a decrease in the extent of incorporation of formate carbon into serine in folic-acid-deficient rats has been reported previously (47).

The mechanism of this inhibitory effect of folic acid deficiency in intact rats is not clear. As far as we know, there are no published indications that folic acid or its derivatives are cofactors in the enzymatic transfer of the methyl group of methionine to either choline or creatine. There are, however, several strong indications that folic acid or its derivatives are cofactors in the synthesis of purines. It is possible, therefore, that the inhibitory effects of folic acid deficiency in intact rats on the transfer reactions involving choline and creatine formation from methionine are a reflection of an indirect interference by the deficiency, possibly via the inhibition of synthesis of certain nucleotides.

These data seem to rule out the participation of vitamin  $B_{12}$  in transmethylation reaction involving methionine. The beneficial effects of dietary vitamin  $B_{12}$  on the growth of rats ingesting diets containing homocystine do not appear to be due to the stimulation of a transfer of the methyl group of synthesized methionine to appropriate acceptors in the organism. This conclusion is in accord with the observations from several laboratories, which have indicated a good growth of rats on diets which were free of vitamin  $B_{12}$  but which contained methionine. This, however, does not by any means prove that vitamin  $B_{12}$  is not essential in the diet of the rat or other species.

The effects of folic acid deficiency on the transfer of the methyl group of methionine to choline and creatine, however, complicate somewhat, though not necessarily fatally, the interpretation of the data on the neogenesis of the methyl groups of choline and creatine from labeled glycine and serine in folic-acid-deficient rats (82). The study of the neogenesis of methionine, along with choline and creatine, however, in this deficiency, makes the task of the interpretation of the earlier data somewhat easier.

VITAMIN B<sub>12</sub> AND FOLIC ACID IN NEOGENESIS  
OF THE METHYL GROUP

Table 8 summarizes the data obtained in our laboratory on the effect of a deficiency in vitamin B<sub>12</sub> in grown rats on the utilization of several labeled metabolites for the synthesis of methionine,

TABLE 8

EFFECT OF DEFICIENCY IN VITAMIN B<sub>12</sub> IN RATS ON THE SYNTHESIS OF METHIONINE,  
CHOLINE, CREATINE, AND SERINE

Isotope administered *	S.S.A. $\times$ 100							
	Normal				Vitamin B <sub>12</sub> -deficient			
	Methio- nine	Trime- thyl- amine	Crea- tine	Serine	Methio- nine	Trime- thyl- amine	Crea- tine	Serine
Formate-C <sup>14</sup>	0.30	1.1	0.2	0.5	0.30	0.8	0.2	0.4
Glycine-2-C <sup>14</sup>	0.20	0.6	0.6	1.1	0.03	0.1	0.4	1.1
Serine-3-C <sup>14</sup>	0.30	1.1	0.3	3.0	0.32	1.2	0.3	3.4
Methionine-CH <sub>3</sub> -C <sup>14</sup>	10.80	38.0	5.2	0.6	10.50	44.3	5.5	0.5

\* Female rats were maintained on a diet containing 8% casein, 0.4% threonine, 0.5% cystine, and 1% glycine. The "normal diet" contained vitamin B<sub>12</sub>. All animals were sacrificed 20 hours after the intraperitoneal injection of a single dose of the isotopes. The metabolites were isolated from the entire carcasses.

choline, creatine, and serine. Under our experimental conditions, only the utilization of the  $\alpha$ -carbon of glycine for the synthesis of the methyl groups of methionine, choline, and creatine was decreased in the vitamin B<sub>12</sub>-deficient intact rat. Contrary to our previously expressed suggestion (82), the deficiency in vitamin B<sub>12</sub> had no appreciable effect on the utilization of the  $\alpha$ -carbon of glycine for serine formation. The inhibition by vitamin B<sub>12</sub> deficiency of the utilization of the  $\alpha$ -carbon of glycine for neogenesis of the methyl group has been recently confirmed, in experiments employing the suckling pig (83).

Arnstein and Neuberger (84), on the other hand, have reported that young rats maintained on vitamin B<sub>12</sub>-free diets for a relatively short time showed a decreased incorporation of the C<sup>14</sup> of formate, glycine-2-C<sup>14</sup>, or serine-3-C<sup>14</sup> into the methyl groups of choline and



methionine. Under their experimental conditions, although not under ours, the extent of incorporation of the  $\alpha$ -carbon of glycine into serine was unaffected by vitamin B<sub>12</sub> deficiency. A detailed discussion of the apparent reasons for our failure to observe an inhibitory effect of vitamin B<sub>12</sub> deficiency on the utilization of formate and the  $\beta$ -carbon of serine for methyl synthesis cannot be given here. Suffice it to say that the diets of some of the rats employed by Arnstein and Neuberger (84) contained choline; ours did not. Arnstein and Neuberger (84) employed weanling rats; we used 2-3 month old rats. The English workers fed their rats the isotopic materials continuously for several days; we injected our materials in a single dose. It appeared to us, therefore, that ample differences in the experimental set-up were present to justify the repetition of the work employing weanling rats, and feeding them diets consisting of a mixture of amino acids containing homocystine. No choline was added to the diet, however. After 60 days on a vitamin B<sub>12</sub>-free diet (several rats died during the period), the surviving animals were repeatedly injected for several days with either glycine-2-C<sup>14</sup>, serine-3-C<sup>14</sup>, formaldehyde-C<sup>14</sup>, or formate-C<sup>14</sup>. As with adult rats, only the utilization of glycine-2-C<sup>14</sup> for methionine and choline formation was significantly decreased in the deficient rat (85). Table 9 summarizes the data obtained in our laboratory on the effect of folic acid deficiency in the rat on the utilization of several labeled metabolites for the synthesis of methionine, choline, creatine, and serine (85).

Under our experimental conditions, folic acid deficiency decreased the utilization of the labeled carbons of formate, glycine, serine, and methionine for choline and creatine synthesis. The synthesis of the methyl group of methionine, isolated from the tissues, was also decreased in the case of glycine-2-C<sup>14</sup> and serine-3-C<sup>14</sup>. The apparent absence of any effect of folic acid deficiency on the extent of utilization of formate for methionine synthesis is, perhaps, a reflection of the combined effect of folic acid deficiency on the synthesis and transfer of the methyl group of methionine to choline and creatine. With this in mind, it is apparent that folic acid deficiency in the rat inhibited the utilization of formate for the synthesis of the methyl



group, if both choline and methionine are taken into account. The deficiency in folic acid, as anticipated from previous work, inhibited the synthesis of serine from all precursors used, with the exception of serine-3-C<sup>14</sup>. It is apparent from the data presented that neither folic acid nor vitamin B<sub>12</sub> deficiency affects the extent of incorporation of either serine or methionine into tissue proteins, under our experimental conditions.

TABLE 9

EFFECT OF FOLIC ACID DEFICIENCY IN RATS ON THE SYNTHESIS OF METHIONINE, CHOLINE, CREATINE, AND SERINE

Isotope administered *	S.S.A. $\times$ 100							
	Normal				Folic-acid-deficient			
	Methio- nine	Trime- thyl- amine	Crea- tine	Serine	Methio- nine	Trime- thyl- amine	Crea- tine	Serine
Formate-C <sup>14</sup>	0.32	2.00	0.3	0.37	0.32	0.48	0.2	0.25
Glycine-2-C <sup>14</sup>	0.20	0.58	0.5	0.80	0.01	0.26	0.3	0.50
Serine-3-C <sup>14</sup>	0.39	3.15	0.5	2.74	0.20	0.30	0.2	3.70
Methionine-CH <sub>3</sub> -C <sup>14</sup>	10.25	31.00	5.0	0.64	12.90	13.00	1.1	0.18

\* Female rats were maintained on a diet containing 8% casein, 0.4% threonine, 0.5% cystine, 1% glycine. "Normal" rats were folic-acid-deficient rats which were administered *citrovorum* Factor ("CF"-Lederle) 3 hours before the isotopes. All animals were sacrificed 20 hours after the intraperitoneal injection of a single dose of the isotopes. The metabolites were isolated from the entire carcasses.

It appeared of interest to extend these studies to other deficiencies, such as the deficiency in pyridoxine or in pantothenic acid. The involvement of pyridoxal phosphate in the metabolic utilization of glycine and serine appeared quite likely, and this might have an indirect effect on the release or the utilization of the C<sub>1</sub> units from these amino acids. A somewhat obscure nutritional relationship between the requirement for pantothenic acid and vitamin B<sub>12</sub> in animals has been previously noted (86). Of greater interest was the observation of Shemin on the possible relationship of his succinyl-glycine cycle to the neogenesis of the methyl group (87). The deficiency in pantothenic acid may thus conceivably affect the utilization of the  $\alpha$ -carbon of glycine for methyl synthesis, if this succinyl-glycine cycle of Shemin plays an important role in the mechanism,



directly or indirectly. On the other hand, failure of pantothenic acid deficiency to affect the utilization of other  $C_1$  donors for methyl synthesis could suggest as a possibility that the succinyl-glycine cycle of Shemin is important in the metabolic transformations of glycine only, including its eventual availability for methyl synthesis, but is not of crucial importance to the mechanisms of utilization of other  $C_1$  precursors, and certainly not to the mechanisms of  $C_1$  transformations to the methyl group. We are aware that these serious possibilities cannot be settled unequivocally by employing an intact animal and that, at best, the results are only of an exploratory nature.

#### PYRIDOXINE DEFICIENCY IN NEOGENESIS OF THE METHYL GROUP

We have previously reported (78) a decreased transfer of the methyl group of methionine to choline and creatine in the pyridoxine-deficient rat. The data summarized in Table 10 show the effect of

TABLE 10

EFFECT OF PYRIDOXINE DEFICIENCY IN RATS ON THE SYNTHESIS OF METHIONINE, CHOLINE, CREATINE, AND SERINE

Isotope administered *	S.S.A. $\times 100$							
	Normal				Vitamin B <sub>6</sub> -free			
	Methio- nine	Trime- thyl- amine	Crea- tine	Serine	Methio- nine	Trime- thyl- amine	Crea- tine	Serine
Formate- $C^{14}$	0.23	0.6	0.2	0.5	0.3	0.8	0.3	0.5
Formaldehyde- $C^{14}$	0.20	0.7	0.2	0.5	0.2	0.9	0.3	0.6
Glycine-2- $C^{14}$	0.08	0.3	1.7	1.4	0.05	0.1	0.6	1.2
Serine-3- $C^{14}$	0.11	0.8	0.3	2.5	0.32	0.5	0.2	2.8
Methionine- $CH_3$ - $C^{14}$	10.5	36.3	4.5	0.4	3.5	13.8	1.7	0.4

\* Female rats were maintained on a diet containing 40% casein. The isotopes were injected intraperitoneally in a single dose, and the animals were sacrificed 20 hours later. The metabolites were isolated from the entire carcasses. "Normal" rats are pyridoxine-deficient rats which were administered pyridoxine 2 days before the isotopes.

this deficiency on the utilization of labeled metabolites for the synthesis of methionine, choline, creatine, and serine in the rat. The utilization of either formate or formaldehyde in the deficient rat for

the synthesis of any of the metabolites which we isolated from the tissues was not apparently affected. The utilization of the  $\alpha$ -carbon of glycine for the synthesis of the methyl groups of methionine, choline, and creatine, as well as for the synthesis of serine, was decreased in the pyridoxine-deficient rat. The incorporation of serine-3- $C^{14}$  into tissue serine was not affected by the deficiency of pyridoxine, whereas the incorporation of methyl-labeled methionine into tissue methionine was. The changes in the extent of incorporation of  $C^{14}$  of serine or glycine into choline and creatine should be considered in the light of the effects produced by pyridoxine deficiency on the utilization of the methyl group of methionine for choline and creatine formation. It would appear from these exploratory data that pyridoxine and its derivatives do not play a decisive role in *the mechanisms of utilization of  $C_1$  units*, once released in the animal organism, for the methyl group formation. The differences in the extent of incorporation of neosynthesized methionine (from formate and formaldehyde) and of administered methionine into tissue proteins in the pyridoxine-deficient rat, reflect some metabolic alterations of administered methionine in this deficiency, to which the neosynthesized methionine is apparently immune. It may also be a reflection of differences in absorption, or of relative dilution of administered methionine by dietary methionine, which the deficient rat failed to metabolize and which, as a result, was present in the deficient-rat tissues in greater concentration than in those of the normal rat.

#### PANTOTHENIC ACID DEFICIENCY IN NEOGENESIS OF THE METHYL GROUP

The data on the utilization of various metabolites for the synthesis of methionine, choline, creatine, and serine in pantothenic-acid-deficient rats are summarized in Table 11. The only significant change produced by the deficiency in pantothenic acid in the rat was a decrease in the utilization of the  $\alpha$ -carbon of glycine for the synthesis of the methyl groups of methionine, choline, and creatine. Significantly, the extent of incorporation of the  $\alpha$ -carbon of glycine into



tissue serine was apparently not affected by pantothenic acid deficiency in the rat.

TABLE 11

EFFECT OF A DEFICIENCY IN PANTOTHENIC ACID IN RATS ON THE UTILIZATION OF FORMATE, FORMALDEHYDE, GLYCINE, SERINE, OR METHIONINE

Isotope administered *	S.S.A. $\times$ 100							
	Normal				Pantothenic-acid-deficient			
	Methio- nine	Trime- thyl- amine	Crea- tine	Serine	Methio- nine	Trime- thyl- amine	Crea- tine	Serine
Formate- $C^{14}$	0.3	1.1	0.2	0.5	0.28	1.03	0.1	0.8
Formaldehyde- $C^{14}$	0.2	0.8	0.2	0.5	0.30	1.19	0.2	0.6
Glycine-2- $C^{14}$	0.2	0.6	0.6	1.1	0.04	0.30	0.4	1.1
Serine-3- $C^{14}$	0.3	1.1	0.3	3.5	0.43	1.50	0.6	4.5
Methionine- $CH_3-C^{14}$	10.8	35.0	5.6	0.6	10.30	33.10	5.0	0.5

\* Female rats were maintained on a diet containing 8% casein, 0.4% threonine, 0.5% cystine, and 1% glycine. The normal diet contained pantothenic acid. All animals were sacrificed 20 hours after the injection of single doses of the isotopes. The metabolites were isolated from the entire carcasses.

These observations are identical with those obtained in our laboratory on vitamin  $B_{12}$ -deficient rats. What the significance is of this metabolic relationship of pantothenic and vitamin  $B_{12}$  deficiencies to glycine utilization cannot be appraised at this stage of experimentation. It is of interest to note, however, that an increase in the concentration of CoA in animal tissues was observed in vitamin  $B_{12}$ -deficient animals (88). The increases were due to both forms of CoA, the disulfide and the sulfhydryl (89), a fact eliminating the possible direct role of vitamin  $B_{12}$  in the reduction of the disulfide bond of CoA. Another interesting observation, recorded in our laboratory (80), was that the extent of acetylation of  $\gamma$ -phenyl- $\alpha$ -aminobutyric acid in the vitamin  $B_{12}$ -deficient rat was reduced. A considerable amount of further work lies ahead to elucidate these relationships, as well as the possible involvement of vitamin  $B_{12}$  and, of course, of pantothenic acid in the utilization of the  $\alpha$ -carbon of glycine for the synthesis of the methyl group, perhaps, via the succinyl-glycine cycle of Shemin.

## POSSIBLE MECHANISMS OF METHIONINE SYNTHESIS

The development of the possibilities stated above in regard to the succinyl-glycine cycle of Shemin in methyl synthesis is awaited with interest. In the meantime, we can do no more than to explore other possibilities in the hope of getting at least a decisive negative answer.

We have previously reported (78) that dietary homocystine in normal rats augmented the extent of incorporation of  $C^{14}$  from formate and from the  $\beta$ -carbon of serine into tissue choline and creatine. Dietary cystine, in lieu of homocystine, was considerably less effective in this respect. The data summarized in Table 12

TABLE 12

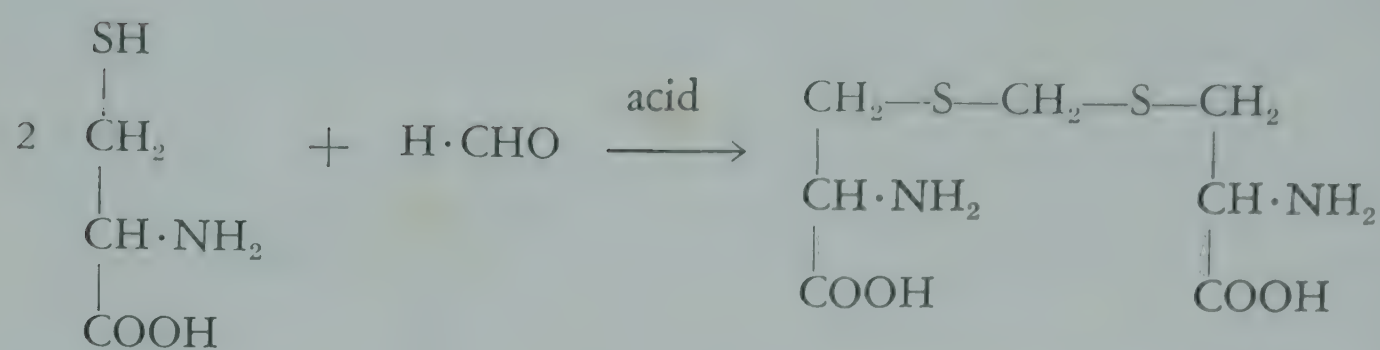
EFFECT OF DIETARY HOMOCYSTINE ON THE SYNTHESIS OF METHIONINE, CHOLINE, CREATINE, AND SERINE IN THE RAT

Isotope administered *	S.S.A. $\times$ 100							
	Homocystine				None			
	Methio- nine	Trime- thyl- amine	Crea- tine	Serine	Methio- nine	Trime- thyl- amine	Crea- tine	Serine
Formate- $C^{14}$	0.35	1.8	0.13	0.7	0.22	0.5	0.1	0.3
Serine-3- $C^{14}$	0.60	2.0	0.50	3.2	0.18	1.2	0.2	2.0

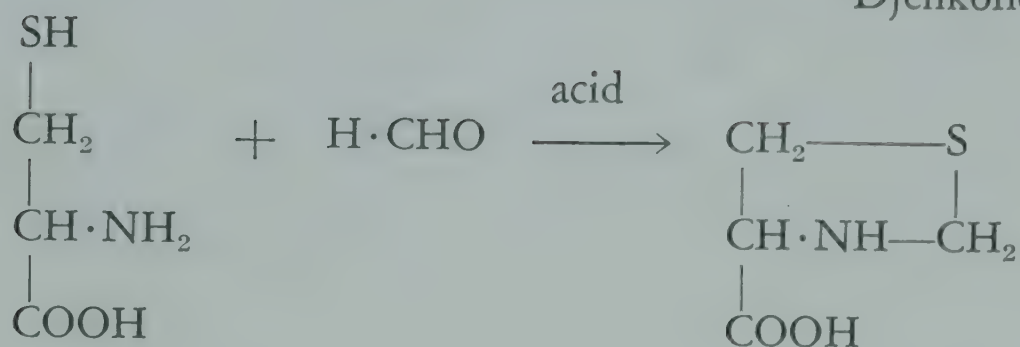
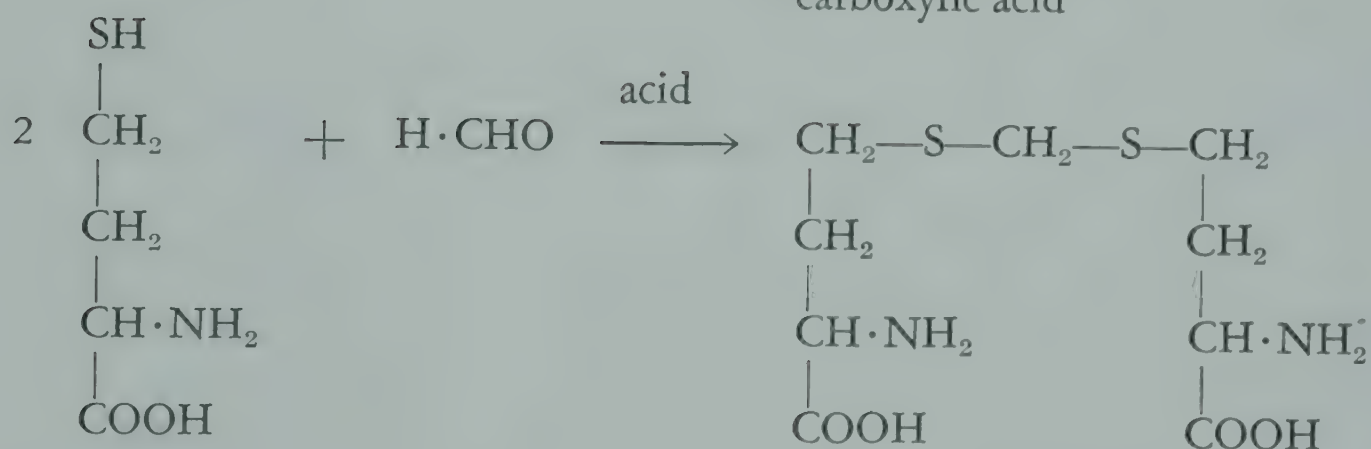
\* Female rats were maintained on amino acid mixture diets, either containing homocystine or free of homocystine. All animals were sacrificed 20 hours after the intraperitoneal injection of a single dose of the isotopes. The metabolites were isolated from the entire carcasses.

illustrate the effect of dietary homocystine on the extent of utilization of formate- $C^{14}$  and of serine-3- $C^{14}$  for the synthesis of methionine, choline, creatine, and serine in normal animals. Homocystine significantly augmented the extent of synthesis of the isolated metabolites from either formate or serine. Similar results were obtained in vitro by Berg (64), who, in addition, recorded that homocysteine augmented the extent of incorporation of  $C^{14}$  of formate into purines. Berg (64) proposed the formation from formate and homocysteine of an intermediate, which acts as a common carrier of  $C_1$  for the synthesis of methionine, serine, and purines via various metabolic





Djenkolic acid

Thiazolidine  
carboxylic acid

Homodjenkolic acid

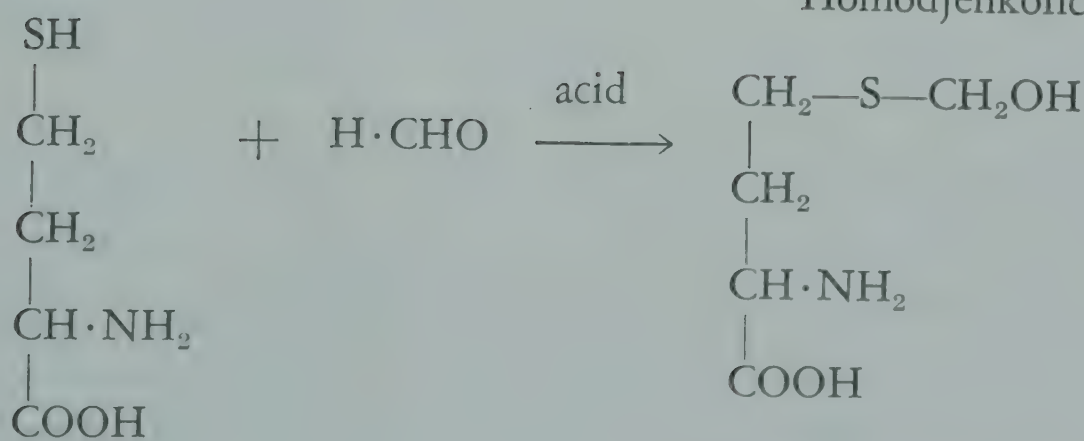
S-hydroxymethyl-  
homocysteine

FIG. 11. The structural relationship of S-hydroxy-methyl-homocysteine to djenkolic acid, thiazolidine carboxylic acid, and homodjenkolic acid.

routes. Apparently, folic acid or its derivatives are involved either in the formation of the intermediate from formate and homocysteine, or in further metabolic transformations of the  $C_1$ -homocysteine intermediate that lead to the formation of methionine, serine, and purines.

The proposed  $C_1$ -homocysteine intermediate, which we designated as "X" in Fig. 1, is S-hydroxymethyl-homocysteine, the possible significance of which in methionine synthesis has been previously discussed by my coworkers and myself on several occasions. Recently, we have synthesized this compound with  $C^{14}$  in the hydroxymethyl group, and have studied its availability for the synthesis of methionine, choline, creatine, and serine, as well as for growth of rats in lieu of methionine in the diet (85). The structural relationship of S-hydroxymethyl-homocysteine to djenkolic acid, thiazolidine carboxylic acid, and to homodjenkolic acid is shown in Fig. 11. The condensation of one or two moles of cysteine with one mole of formaldehyde to give thiazolidine carboxylic acid or djenkolic acid has been studied by Armstrong and du Vigneaud (90). Dyer (33) reported a preliminary study in which homodjenkolic acid has been prepared by an analogous procedure, and she reported that homodjenkolic is unavailable to rats for growth in lieu of methionine.

Growth studies in our laboratory have indicated that homodjenkolic acid, as reported by Dyer (33), is unavailable to rats, while S-hydroxymethyl-homocysteine is available. The tracer data on S-hydroxymethyl-homocysteine- $CH_2OH-C^{14}$  are recorded in Table 13. The extent of incorporation of  $C^{14}$  of S-hydroxymethyl-homocysteine into tissue methionine, choline, creatine, and serine was greater than that obtained from labeled formate or formaldehyde, glycine-2- $C^{14}$ , or serine-3- $C^{14}$ . However, as could be expected, perhaps, methionine- $CH_3-C^{14}$  was much more effective than S-hydroxymethyl-homocysteine in the synthesis of tissue choline, creatine, and methionine, although not of tissue serine. Whether or not S-hydroxymethyl-homocysteine is the, or an, intermediate in the utilization of  $C_1$  for the synthesis of methionine, etc., cannot be either affirmed or denied on the basis of these or other data. Further work is required to establish its formation in biological systems, to test the lability of the S-hydroxy-



methyl group in the biological environment, etc. It should be pointed out, however, that the compound is stable in a strong acid medium.

TABLE 13

THE UTILIZATION OF S-HYDROXYMETHYL-HOMOCYSTEINE IN RATS

Isotope administered *	S.S.A. $\times 100$			
	Methio- nine	Trimethyl- amine	Creatine	Serine
Methionine-CH <sub>3</sub> -C <sup>14</sup>	11.5	30.0	6.4	0.55
S-Hydroxymethyl-homocysteine-CH <sub>2</sub> OH-C <sup>14</sup>	1.1	3.8	0.8	1.40
Formaldehyde-C <sup>14</sup>	0.3	0.7	0.2	0.24
Formate-C <sup>14</sup>	0.3	0.7	0.1	0.32
Glycine-2-C <sup>14</sup>	0.2	0.5	0.5	1.00
Serine-3-C <sup>14</sup>	0.3	1.0	0.3	2.70

\* Adult female rats were maintained on a diet containing 8% casein, 0.4% threonine, 0.5% cystine, and 1% glycine. All animals were sacrificed 20 hours after the intraperitoneal injection, in a single dose, of the isotopes. The metabolites were isolated from the entire carcasses.

### TRANSMETHIOLATION

Of great interest is the utilization of thiomethyladenosine in the biosynthesis of methionine by a mutant culture 68 of *Aerobacter aerogenes* in the presence of  $\alpha$ -aminobutyric acid (91). At least 80 per cent of the thiomethyl group of methionine produced by the mutant originated from thiomethyladenosine-CH<sub>3</sub>-C<sup>14</sup>-S<sup>35</sup>. During growth on the thiomethyladenosine the mutant produced adenine. Homocysteine did not increase the rate of growth beyond 0.1  $\mu$ moles per ml. of medium, at which the growth was only half maximal. The possible route of methionine synthesis in this mutant is apparently the reverse of methionine degradation in yeast and possibly in animals. The reactions are shown in Fig. 12.

Since  $\alpha$ -aminobutyric acid is formed in animals from methionine, it becomes of some interest to ascertain if thiomethyladenosine and aminobutyric acid could give rise to methionine in animals, or if thiomethyladenosine can actually arise from methionine. The formation of methyl mercaptan from methionine in vitro has been recorded

(92). The carbon of methyl mercaptan has been recovered in the methyl group of methionine in vivo (93). The latter results were attributed to the oxidation of the mercaptan, possibly to formate which then entered the methyl group of methionine, by analogy with the possible incorporation of the carbon of methanol into the

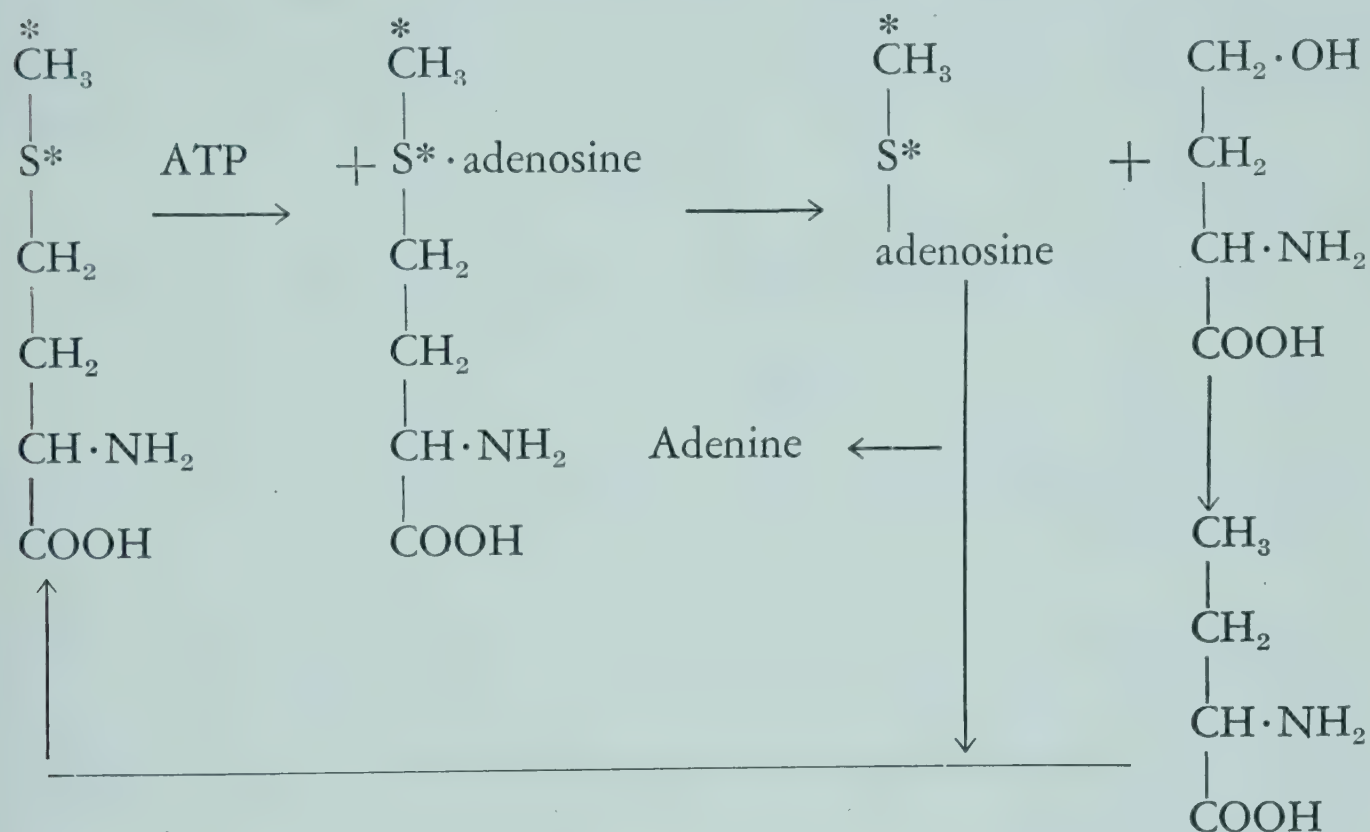


FIG. 12. The pathway of methionine synthesis in *Aerobacter aerogenes* mut. 68.

methyl group (94). The interpretation of the results with methyl mercaptan were supported by the observation that the sulfur of the mercaptan, labeled with  $\text{S}^{35}$ , was not recovered in methionine, but has undergone oxidation to sulfate.

Schlenk and Tillotson (95) reported the utilization of methyl mercaptan by yeast cultures in the synthesis of methionine, which, in turn, stimulated the synthesis of adenosylmethionine and thio-methyladenosine. These reactions are shown schematically in Fig. 13.

Schlenk and Tillotson (95) found that in yeast the reaction is not confined to methyl mercaptan, as ethyl mercaptan was also utilized by yeast in the formation of thioethyladenosine via the intermediate formation of ethionine and adenosylethionine. Ethionine, fed to yeast, yielded thioethyladenosine. Referring to the observations of Neuberg (96) that acetaldehyde reacts with hydrogen sulfide in



fermenting yeast to give ethyl mercaptan, Schlenk and Tillotson (95) suggested the biological formation of ethionine, which, heretofore, has been considered an artifact.

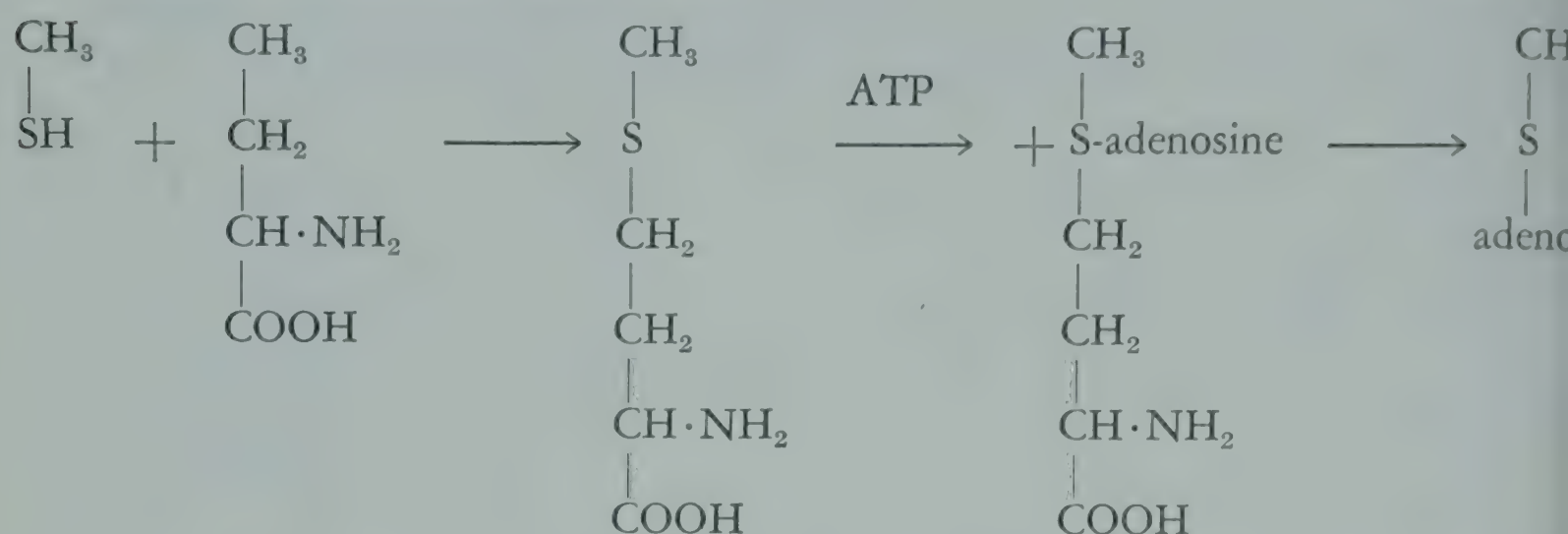


FIG. 13. Utilization of methyl mercaptan by yeast cells in the synthesis of adenosyl-methionine and thiomethyladenosine. (F. Schlenk and J. A. Tillotson, *Federation Proc.* 13, 290, 1954).

It thus appears clear that the interaction with ATP is not confined to methionine, that the biological formation of thioadenosyl compounds is of greater biological significance, and that their formation is of importance not only in the degradative pathways of methionine alone, but also in the synthetic pathways leading to methionine and related products. It is well within possibility that, in animals, methionine undergoes a partial degradation to thiomethyladenosine, which is then reutilized in situ for the resynthesis of methionine. This possibility poses the question of the origin of the aminobutyric acid moiety of methionine in this synthesis, a problem at present under study in our laboratory.

#### SYNTHESIS OF METHIONINE BY TRANSMETHYLATION VERSUS NEOGENESIS

In the course of the presentation of the data recorded in Tables 6 and 7 we pointed out the relatively low incorporation of the methyl group of choline into tissue methionine of rats, under our experimental conditions. The methyl group of betaine, on the other hand, was considerably more effective in this respect, as, perhaps, could

be expected. Before the transfer of the methyl group of choline to methionine can occur, choline is expected to be oxidized to betaine through the mediation of systems known collectively as "choline oxidase." The *in vivo* data, therefore, could be interpreted as a reflection of relatively low "choline oxidase" activity in our animals. On the other hand, it could also be interpreted as a result of a "dilution" of administered choline by body choline, or as a reflection of both factors.

TABLE 14

THE EFFECT OF DIETARY CHOLINE OR BETAINES ON THE UTILIZATION OF HOMOCYSTINE OR METHIONINE FOR THE SYNTHESIS OF METHIONINE OR CHOLINE IN THE RAT

Supplement to diet *	Isotope administered	S.S.A. $\times$ 100		
		Choline	Creatine	Methionine
None	Homocystine-2-C <sup>14</sup>			9.5
Choline	"			9.1
Betaine	"			9.1
None	Methionine-2-C <sup>14</sup>			17.1
Choline	"			16.9
Betaine	"			16.8
None	Methionine-CH <sub>3</sub> -C <sup>14</sup>	31.0	4.0	7.9
Choline	"	17.9	3.2	7.8
Betaine	"	16.1	4.2	6.6
Methionine	"	24.0	2.2	4.2

\* Adult female rats were maintained on amino-acid-mixture diets, containing homocystine, or the same diet supplemented with choline or betaine. Methionine replaced homocystine. All animals were sacrificed 20 hours after the intraperitoneal injection of a single dose of the isotopes. The metabolites were isolated from the entire carcasses.

If the extent of oxidation of choline in the rat to betaine is high, and our data only reflect the dilution of administered choline by body choline, then one could expect dietary non-isotopic choline to dilute out the methyl group of C<sup>14</sup>-labeled methionine administered to rats at the same time. The promotion of growth in rats by dietary choline, fed to them along with homocystine, was also attributed to an increased synthesis of methionine from the methyl group of choline. The data shown in Table 14 indicate that the extent of incorporation of homocystine-2-C<sup>14</sup> into tissue methionine of rats



was not increased by feeding either choline or betaine; neither was the extent of incorporation of methionine-2-C<sup>14</sup> into tissue methionine affected by feeding either choline or betaine. Feeding of choline to rats receiving methionine-CH<sub>3</sub>-C<sup>14</sup> did not affect the extent of incorporation of the labeled carbon of methionine into tissue methionine, while either betaine or, more significantly, methionine, did so. Dietary choline, betaine, or methionine decreased the extent of incorporation of the methyl group of radiomethionine into choline. It is logical to assume that dietary choline will spare the rat the necessity of elaborating choline via transmethylation from methionine. The data may indicate this possibility, but they also may be a reflection of at least two factors which operate in the animal: the dilution of the synthesized choline by dietary non-isotopic choline, and the increased rate of turnover of the synthesized choline because of feeding choline in the diet. We observed in our rats a many-fold increase in the extent of oxidation of the methyl-labeled choline to respiratory CO<sub>2</sub> upon feeding them diets containing choline. At the same time, the turnover of tissue choline was increased several fold. Thus we cannot state unequivocally that such data as ours and those of others prove that dietary choline "spares" the animal the need for synthesizing the methyl group of methionine de novo, and that in the presence of dietary choline this synthesis of methionine de novo is quantitatively insignificant.

The data in Table 15 show comparative results, obtained in weanling rats which, along with a complete amino-acid-mixture diet containing homocystine, were fed radioactive choline, betaine, formate, serine, or methionine for a period of 15 days. The data are expressed as the relative specific activities of methionine and choline, isolated from the tissues at the end of the feeding period (85). The same relative inefficiency of the methyl group of choline for methionine synthesis is indicated. In appraising the quantitative significance of the extent of synthesis of the methionine methyl group from serine and formate, one confronts the difficulty of taking into account the relative size of the "formate pool," which undoubtedly is generously supplied from a variety of C<sub>1</sub>-precursors, and

which will affect the extent of incorporation of the labeled carbons of formate or serine into the methyl group of methionine. In other experiments, which we will not detail here, we observed that dietary choline did not significantly affect the extent of synthesis of the methyl group of methionine from radio-labeled formate, glycine,

TABLE 15

SYNTHESIS OF TISSUE CHOLINE AND METHIONINE IN GROWING RATS FROM CHOLINE-CH<sub>3</sub>-C<sup>14</sup>, BETAINE-CH<sub>3</sub>-C<sup>14</sup>, OR METHIONINE-CH<sub>3</sub>-C<sup>14</sup>, SERINE-3-C<sup>14</sup> OR FORMATE-C<sup>14</sup>

Dietary Isotope fed for 15 days, 0.4 mM./100 g./day	Relative Specific Activity × 100	
	Methionine	Choline
Choline-CH <sub>3</sub> -C <sup>14</sup>	4.0	100.0
Betaine-CH <sub>3</sub> -C <sup>14</sup>	10.0	44.0
Methionine-CH <sub>3</sub> -C <sup>14</sup>	26.0	63.1
Serine-3-C <sup>14</sup>	1.8	3.5
Formate-C <sup>14</sup>	0.9	1.4

Diet: Complete amino acid mixtures, containing vitamin B<sub>12</sub> and folic acid, free of choline, methionine, cystine, glycine, or serine. In the absence of methionine-CH<sub>3</sub>-C<sup>14</sup>, all diets contained homocystine.

or serine in the growing rat (80). Clearly, the neogenesis of the methyl group of methionine in the growing animal continues unsuppressed in the presence in the diet of such "methyl donors" as choline.

The rapidity and the extent of the transfer of the methyl group to and from methionine in vitro and in vivo, and the "antilipogenic" and anti-hemorrhagic kidney effect of methionine in vivo are strong indications of the reality of the transmethylation reactions in vivo, and they undoubtedly constitute the major reactions in the degradative and synthetic pathways of methionine. This conclusion will probably remain even if deuterium, as a label of the methyl group, will prove to be not an unequivocal tool in the study of the transmethylation reactions involving methionine.



## RECAPITULATION

From the degradative reactions of methionine in vivo, several major synthetic pathways are initiated: the synthesis of cysteine; the transfer of the methyl group to various acceptors; the oxidation of the methyl group to a  $C_1$  component or components and their subsequent utilization; and the synthesis of aminobutyric acid or its derivatives from the carbon chain of methionine. Most of these reactions are either directly or indirectly reversible, the extent of the reversibility of a particular reaction varying with the species of organism. The synthesis of methionine in vivo appears to follow at least five different pathways: (1) by direct transfer of the methyl group from N-onium compounds such as betaine; (2) by direct transfer of the methyl group from S-onium compounds such as the thietins; (3) by the process of transmethylation involving thio-methyladenosine and aminobutyric acid; (4) by the reversal of the synthetic pathway of cysteine from methionine; and (5) by synthesis de novo of the methyl group of methionine via the process of utilization of the  $C_1$  units, originating from numerous metabolites via various paths, including the oxidation of the methyl group of methionine and its subsequent reduction.

Vitamin  $B_{12}$  does not appear to be involved in any of the processes of methionine formation with the exception of synthesis of the methyl group de novo from the  $\alpha$ -carbon of glycine. The nature of this involvement of vitamin  $B_{12}$  appears to be indirect, since the deficiencies in either folic acid, pyridoxine, or pantothenic acid in rats similarly affected the extent of synthesis of the methyl group of methionine from the  $\alpha$ -carbon of glycine. It thus appears that, in the intact animal, at least four cofactors lie in the pathway of methionine synthesis from glycine: vitamin  $B_{12}$ , folic acid, pyridoxine, and pantothenic acid.

The involvement of folic acid in the synthesis of purines from  $C_1$  fragments, suggests a role of this cofactor in the synthesis of purine and, possibly, pyrimidine nucleotides in intact animals. The inhibitory effect of a deficiency in folic acid in intact animals on the

transfer of the methyl group of methionine to various acceptors may have been a result of the interference by the deficiency in folic acid with nucleotide formation.

A detailed further study of the mechanisms of the metabolic machinery concerned with some of the synthetic pathways discussed here will disclose the exact roles, if any, of the cofactors, and their relationships in the metabolic net of intact animals.

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# ENZYMATIC PATHWAYS IN THE DEGRADATION OF SULFUR-CONTAINING AMINO ACIDS<sup>1</sup>

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## INTRODUCTION

THE CATABOLISM of the sulfur-containing amino acids may be conveniently subdivided into four topics:

- (1) the metabolic transformations of methionine and homocysteine, and their conversion to cysteine;
- (2) non-oxidative reactions of cysteine;
- (3) the oxidation of cysteine and cystine; and
- (4) the enzymatic reactions of cysteinesulfinic and cysteic acids and the formation of inorganic sulfate.

The extensiveness of the literature dealing with the intermediary metabolism of these amino acids does not permit a full discussion of all these topics within the confines of this review. Consequently, the first three of these topics will be surveyed relatively briefly. The metabolic transformations of methionine and homocysteine and the formation of cysteine have been extensively reviewed (1, 2, 3), and certain aspects of this problem, as well as of the second topic, have been covered in the preceding discussion. Regarding the enzymatic oxidation of cysteine and cystine, knowledge remains too scanty to warrant a detailed discussion. The fourth topic has been the subject of extensive investigations in recent years, and, since much of the material is still unpublished, a more detailed consideration of this aspect of the problem will be attempted.

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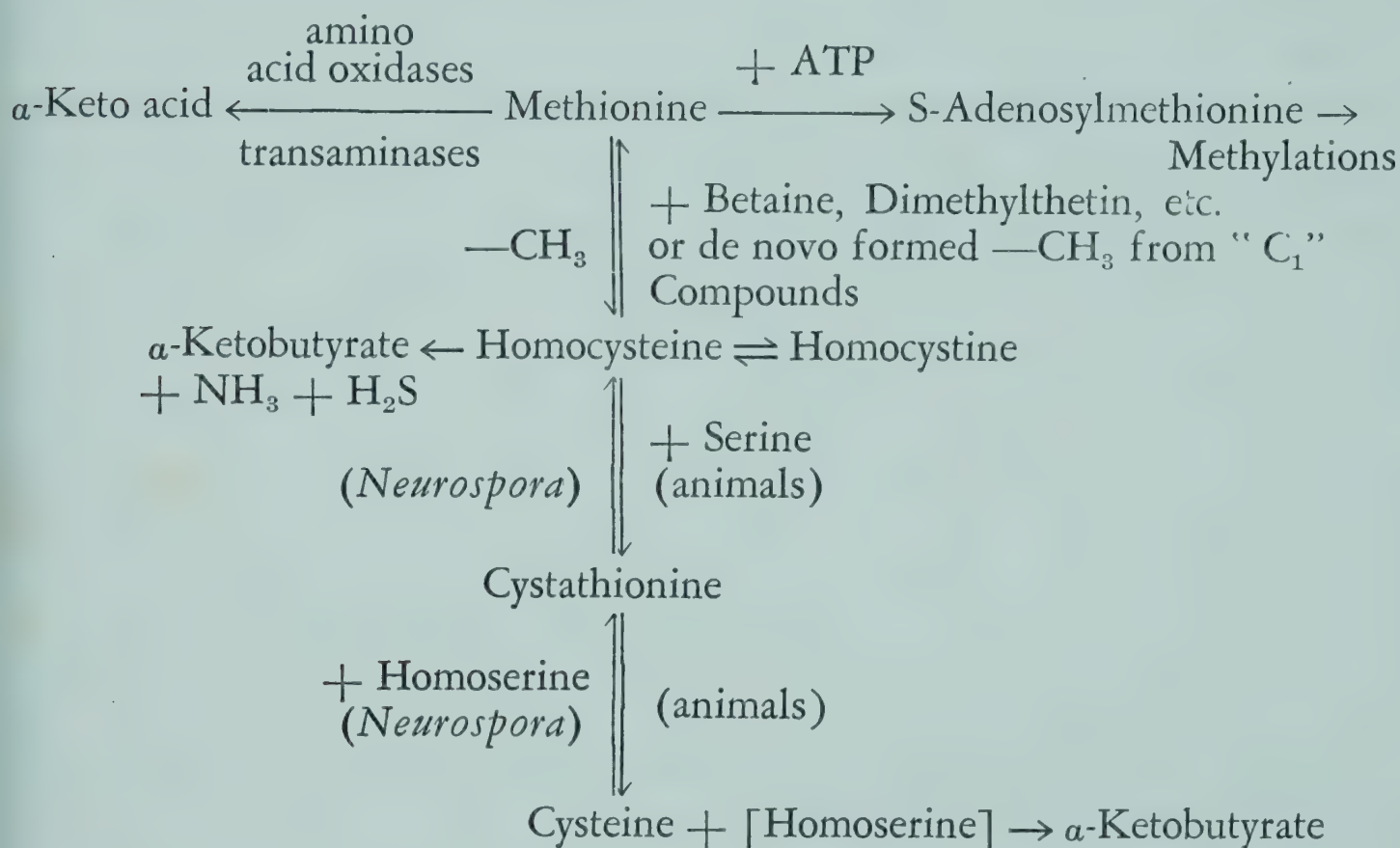
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# METABOLIC TRANSFORMATIONS OF METHIONINE AND HOMOCYSTEINE

The main metabolic reactions of methionine and homocysteine are summarized in Scheme 1. From the now classical investigations of duVigneaud and coworkers (1, 4, 5) and of Borsook and Dubnoff

## SCHEME 1

### METABOLIC REACTIONS OF METHIONINE AND HOMOCYSTEINE



(6, 7) it is known that in the animal body methionine synthesis involves the methylation of homocysteine by betaine, dimethylthetin, or by methyl groups formed de novo. As just discussed by Dr. Cantoni, the primary acceptor may be S-adenosylhomocysteine in certain cases. The catabolism of methionine may occur by a variety of routes. The L-isomer is transaminated in animal tissues to the corresponding  $\alpha$ -keto acid (8) and it is rapidly oxidized to the same product by the L-amino acid oxidase of rat kidney and liver (9), of snake venoms (10, 11), and of *Neurospora* (10); the D-isomer is attacked by the D-amino acid oxidases of kidney (10) and of *N. crassa* (10, 12).



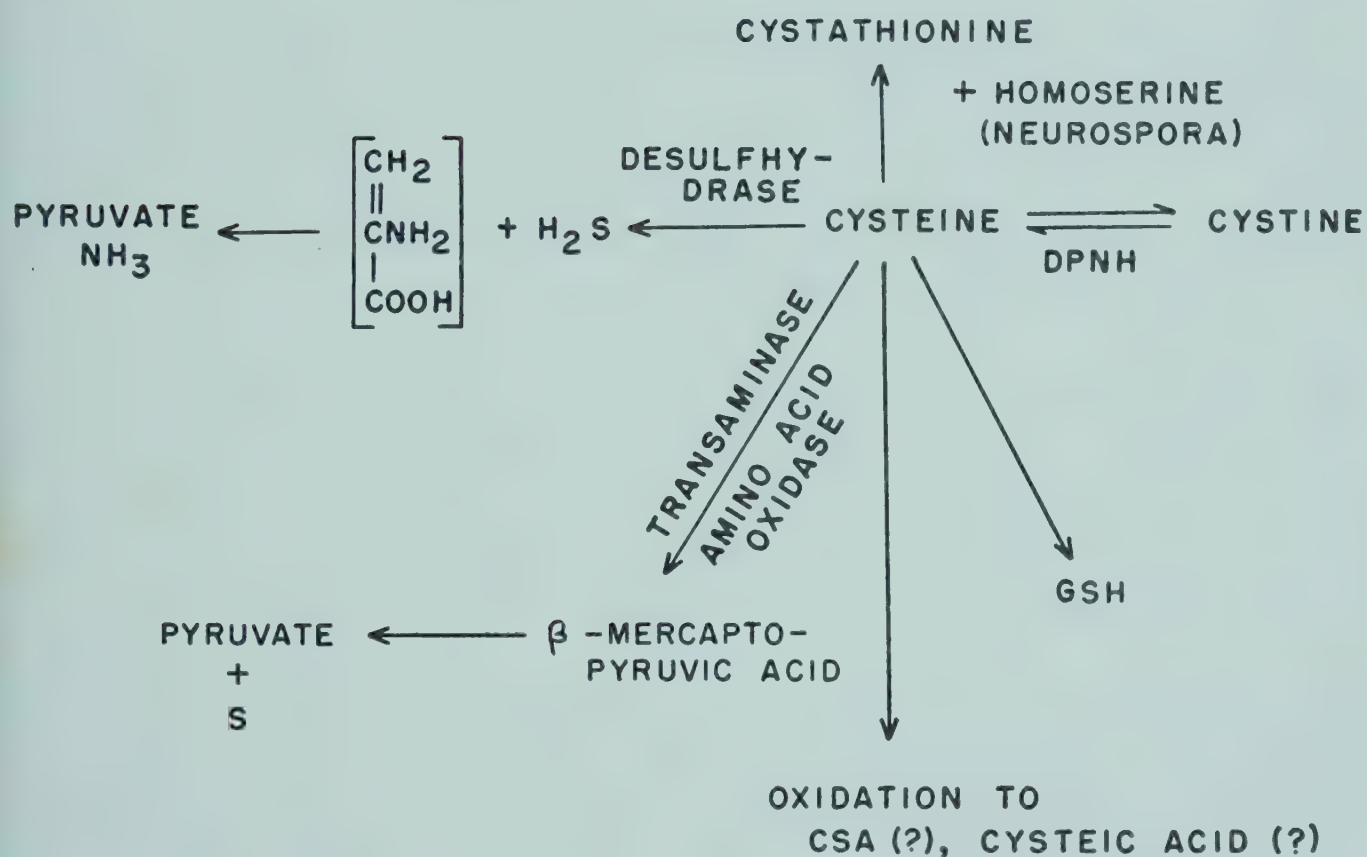
The most widely investigated biological function of methionine is its participation in a variety of methylation reactions. From the work of Cantoni (13), it is known that at least in some of these transmethylations the active form of methionine is S-adenosylmethionine, which is formed from the amino acid by a reaction requiring ATP. As indicated by the results of Smith and Schlenk (14, 15), the same compound is likely to be the precursor of thiomethyladenosine, which also functions as a methyl transfer agent, at least in certain microorganisms (16).

From nutritional studies and from isotope experiments (17-20), it has been known for many years that the sulfur of methionine is utilized in the biogenesis of cysteine, whereas the carbon chain of the latter arises from serine. The pathway of conversion of methionine to cysteine has been considerably clarified by the studies of duVigneaud, Binkley, Horowitz, and their collaborators (19, 21-26) on cystathionine synthesis and breakdown in animal tissues and in mutants of *Neurospora*. The conversion probably occurs by demethylation of methionine to homocysteine, condensation of homocysteine with serine under the influence of the cystathionine-synthesizing enzyme (24, 26), and cleavage of L-cystathionine to cysteine and a second fragment. This fragment may be homoserine but it is converted to  $\alpha$ -ketobutyrate in the enzyme preparations (24, 27). In *Neurospora*, the formation of L-cystathionine involves the condensation of homoserine and cysteine (22).

The formation of  $\alpha$ -ketobutyrate from homocysteine may also occur by another route. As first demonstrated by Fromageot and Desnuelle (28), homogenates of a variety of animal tissues liberate  $H_2S$  from homocysteine. While the formation of  $\alpha$ -ketobutyrate was not demonstrated in this case, an analogous reaction in cell-free extracts of *Proteus morganii* has been shown by Kallio (29) to involve the stoichiometric conversion of homocysteine to  $\alpha$ -ketobutyrate,  $NH_3$ , and  $H_2S$ . Although the reaction mechanism has not yet been elucidated, the intermediate formation of 2-amino-3-enebutyric acid appears probable.

## ANAEROBIC REACTIONS OF CYSTEINE

*Cysteine desulphydrase* (*cysteinase*, *desulfurase*) (Scheme 2). The conversion of cysteine sulfur to  $\text{H}_2\text{S}$  in bacteria has been known for half a century. The analogous reaction in animal tissues was first

SCHEME 2METABOLIC REACTIONS OF CYSTEINE AND CYSTINE

demonstrated in Fromageot's laboratory in 1939 (30). Shortly thereafter, Smythe (31) found that, along with  $\text{H}_2\text{S}$ , the reaction produced  $\text{NH}_3$  and pyruvate. Imperfect analytical methods and the occurrence of interfering and competing enzymatic reactions in the crude preparations prevented the experimental demonstration of the stoichiometry of the reaction for a long time. Thus, the amount of pyruvate formed was always much less than the amount of  $\text{H}_2\text{S}$  and  $\text{NH}_3$ , whereas the disappearance of cysteine exceeded the formation of  $\text{H}_2\text{S}$  (31, 32). At present it is generally agreed (33), however, that the overall reaction is





The formation of aminoacrylic acid in the primary enzymatic reaction (reaction 2) was considered earlier by Smythe (31, 32) and by Green and Stumpf (34) and has been rendered extremely likely by the discovery (35-37) that the desulfhydrase reaction is pyridoxal-phosphate-dependent. This fact has strengthened the analogy with the action of serine dehydrase, an enzyme long considered to bring about the intermediate formation of an  $\alpha,\beta$ -unsaturated linkage by dehydration.

In the light of current concepts on the mechanism of vitamin B<sub>6</sub>-catalyzed reactions (38), the circumstance that Metzler and Snell (39) have been able to reproduce the cysteine desulfhydrase reaction in a model system with a pyridoxal-metal chelate catalyst strengthens the view that aminoacrylic acid is the first product and that the spontaneous rearrangement and hydrolysis of this compound gives rise to pyruvate and NH<sub>3</sub> (reactions 2-4).



In earlier studies on the cysteine desulfhydrase reaction, the accumulation of alanine (30) and the excessive disappearance of cysteine (32) were observed, phenomena which cannot be accounted for by the reaction mechanism described. The most likely explanation of these discrepancies appears to be the presence in these preparations of contaminating transaminases, which may have brought about the transamination of cysteine with pyruvate (either directly or by double transamination with a trace of  $\alpha$ -ketoglutarate) to yield alanine and  $\beta$ -mercaptopyruvate. The latter compound <sup>2</sup> may have escaped detection in the analytical method employed by Smythe (31).

<sup>2</sup> The formation of cystine, observed by both Fromageot et al. (30) and by Smythe (31) may have been due to the presence of  $\beta$ -mercaptopyruvate desulfurase (reaction 5). The free sulfur would then react



with excess cysteine (reaction 6) to give cystine:





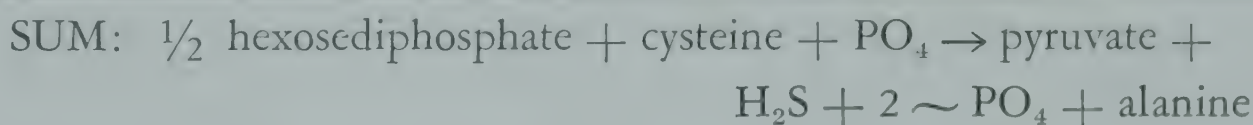
*Transamination.* Cysteine has been shown by Cammarata and Cohen to be transaminated with  $\alpha$ -ketoglutarate in the presence of heart and liver preparations (8). The product of the transamination,  $\beta$ -mercaptopyruvate, would also arise from the aerobic oxidation of cysteine by amino acid oxidases. L-Cysteine is moderately slowly acted upon by the oxidase of certain snake venoms (40), but there are no reliable values in the literature on the rate of oxidation of cysteine by other oxidases. L-Cystine is oxidized by the enzyme from rat kidney (9) as well as by its counterpart from *N. crassa* (10).

*The fate of  $\beta$ -mercaptopyruvate.* The  $\alpha$ -keto acid produced by these reactions appears to be metabolically a highly active compound. As recently shown by Meister and his colleagues (41), preparations from a large array of animal tissues, as well as some bacteria, convert this compound to pyruvate and elemental sulfur (see also 42). The latter can be trapped as  $H_2S$ , if an excess of a thiol compound is present. The enzyme concerned is not identical with cysteine desulfhydrase. Thus cysteine may be converted to pyruvate in animal tissues and bacteria by at least two anaerobic routes: by the desulfhydrase reaction and by transamination, with subsequent desulfuration of the keto acid.

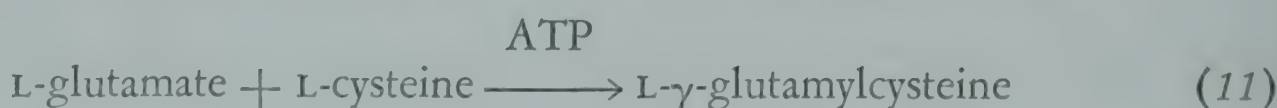
This remarkable reaction may well be the clue to the interdependence of carbohydrate metabolism and  $H_2S$  production from cysteine which has been observed in certain microorganisms. Thus, for example, Kun (43) has recently demonstrated that in intact cells and in cell-free preparations from *Endamoeba histolytica* the anaerobic fermentation of carbohydrates and  $H_2S$  formation from cysteine are completely interdependent reactions. Besides this dependence of  $H_2S$  formation on carbohydrate fermentation, the reaction is further distinguished from typical desulfhydrase action by the fact that alanine, rather than  $NH_3$ , is produced (44). According to Kun (43), the results indicate that thiol radicals are first liberated from the sulfur compound and that their reduction to  $H_2S$  is coupled with the oxidation of triose phosphate to diphosphoglycerate. A possible interpretation of these observations, admittedly speculative, may be as follows.



The first reaction (7) is a transamination between cysteine and pyruvate, directly or by a double transamination involving  $\alpha$ -ketoglutarate, the net result being the formation of a mole each of alanine and  $\beta$ -mercaptopyruvate. The next step (8) may be the desulfuration of the latter, with the formation of S and the regeneration of pyruvate. The S thus formed may then be reduced to  $H_2S$  at the expense of the oxidation of triose phosphate (the latter arising from hexosediphosphate) (reaction 9). In the last step (10), diphosphoglycerate would be converted to pyruvate.



*Glutathione Synthesis.* A further non-oxidative reaction of cysteine which is of considerable interest is its conversion to glutathione. As clearly demonstrated by Bloch and coworkers (45), the synthesis of GSH in pigeon liver extracts proceeds by the formation of L- $\gamma$ -glutamylcysteine, a reaction requiring ATP (reaction 11).



Inasmuch as the subject was reviewed in extenso at these symposia two years ago, more detailed consideration does not seem to be warranted at this time.

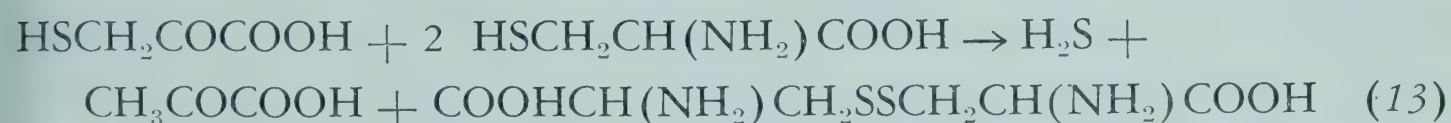
### ENZYMATIC OXIDATION OF CYSTEINE

*Cysteine  $\rightleftharpoons$  Cystine Interconversions.* The best known enzymatic reaction for the oxidation of the thiol group of cysteine to the disulfide form is that catalysed by cytochrome *c* and cytochrome oxidase (46). The reaction is inhibited by known poisons of cytochrome oxidase. A second means may be the rapid, spontaneous oxidation

of cysteine by elemental sulfur to cystine, at the expense of the reduction of S to H<sub>2</sub>S (reaction 12).



As shown by Meister et al. (41), this reaction may be utilized to trap the S arising from the enzymatic desulfuration of  $\beta$ -mercapto-pyruvate. Under these conditions coupled reaction (13) may be obtained.



The reduction of cystine to cysteine by DPNH (reaction 14) has been demonstrated with extracts of baker's yeast and of higher plants by Nickerson and Romano (47).



The reaction is analogous to that of glutathione-TPNH reductase (48-50), except that both types of reductases are specific with respect to the thiol as well as the pyridine nucleotide reactants.

*Oxidation of Cysteine to Cysteinesulfinic Acid.* The enzymatic interconversions of cysteine and cystine and the known metabolic equivalence of these two amino acids in vivo seem to justify the traditional point of view that there exists a common pathway for their oxidation. Indeed, there is reason to believe that L-cystine is reduced to L-cysteine prior to oxidation of the carbon chain or of the S moiety (51), although the existence of an alternate oxidative pathway in which cystine is the initial reactant cannot be precluded (52).

The initial stages in the oxidation of these two amino acids remain almost entirely obscure. The few in vitro investigations that have been focused on this phase of metabolism date back to the late 1930's and early 40's. The unavailability of sufficiently specific analytical methods at the time and the scarcity of information on the metabolism of related amino acids offered little chance for definitive conclusions on either the metabolic pathways or on the nature of the enzymes involved.



Outstanding among these early investigations was the work of Pirie (53), who demonstrated the formation of inorganic sulfate from cysteine, cystine, and methionine in tissue slices. Pirie postulated that both cysteine and cystine are oxidized to cysteinesulfinic acid with the intermediate formation of the hypothetical sulfenic acid, and that the sulfinic acid was the chief site of mineralization of organic sulfur. Thus sulfate arose from inorganic sulfite and not from cysteic acid. Pirie's ideas on the origin of cysteine sulfinic acid remain to this day a feasible working hypothesis; and as to the origin of inorganic sulfate, recent research has proven the validity of this hypothesis, as will be discussed presently.

In 1939 Bernheim and Bernheim (54), using a ground, washed liver preparation, observed rapid oxidation of L-cysteine with the uptake of 3 atoms of  $O_2$ . This cysteine/ $O_2$  uptake ratio led Bernheim and Bernheim to postulate that cysteic acid was the end-product, although its formation was not demonstrated, and in the light of present knowledge cysteic acid is unlikely to be the main end-product of the oxidation of cysteine in liver. These observations were confirmed and extended by Medes and Floyd (52, 55), who also showed that cysteine sulfinic acid is rapidly oxidized and converted to inorganic sulfate in cell-free preparations, and thereby strengthened the view that cysteine sulfinic acid is on the main pathway of oxidation of cysteine and cystine.

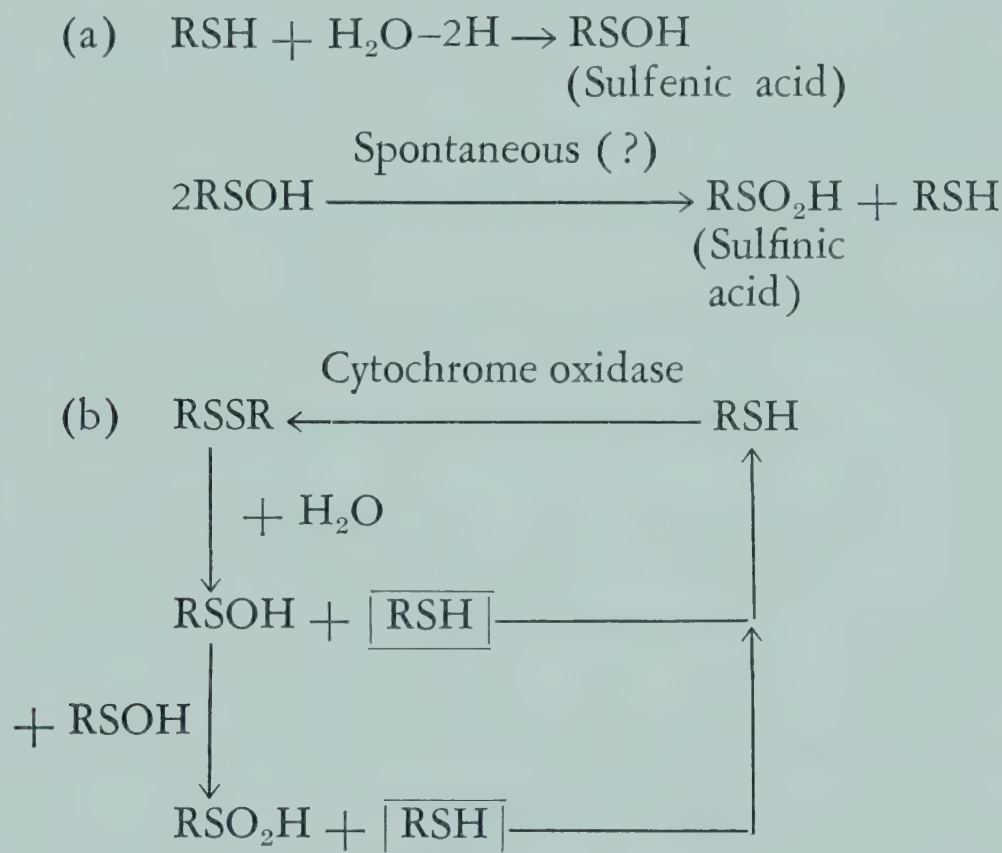
The accompanying diagram (Scheme 3) summarizes the hypotheses of Pirie and of Medes and Floyd on the origin of cysteine sulfinic acid. Cysteine is believed to be enzymatically oxidized to cysteine sulfenic acid; the latter then undergoes a spontaneous dismutation to cysteine sulfinic acid and cysteine. Alternatively, cystine may be visualized as undergoing enzymatic hydrolysis to 1 mole each of the sulfenic acid and of cysteine; the sulfenic acid then undergoes the dismutation to the sulfinic acid and cysteine, discussed above.

The postulated role of cysteine sulfinic acid in the oxidation of cysteine has found considerable support in recent years by the discovery of the wide array of rapid metabolic reactions in which cysteine sulfinic acid participates (56-58) and by the demonstration (59)

that cysteine injected into rats is converted to hypotaurine, a previously known decarboxylation product of cysteine sulfinic (60). The problem of the enzymatic pathway from cysteine to cysteine sulfinic appears to be ripe for reexamination with the aid of the sensitive and specific micromethods of analysis now available.

### SCHEME 3

SOME POSTULATED ROUTES FOR THE INITIAL STAGES OF THE OXIDATION OF CYSTEINE AND CYSTINE (Pirie, Medes, and Floyd)



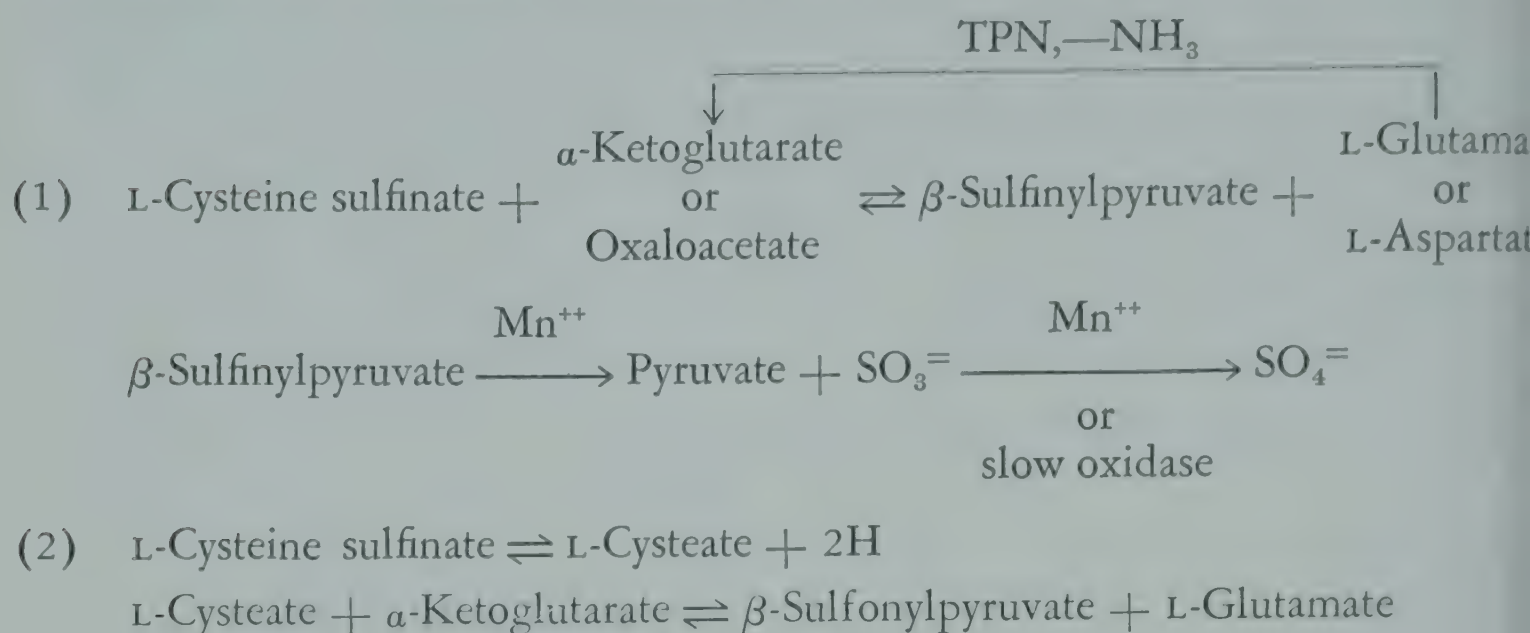
### INTERMEDIARY METABOLISM OF CYSTEINE SULFINIC AND CYSTEIC ACIDS AND THE FORMATION OF SULFATE

*Proteus vulgaris*. Chronologically, *Proteus vulgaris* was the first organism whose metabolic activities toward cysteine sulfinic were systematically surveyed (57, 61, 62). The results of this investigation are briefly summarized in Scheme 4. We found that in intact cells of the organism both L-cysteine sulfinic and L-cysteate are rapidly oxidized. The oxidation of cysteine sulfinic is extensive and results in the formation of  $\text{CO}_2$ ,  $\text{NH}_3$ , and  $\text{SO}_4^{=}$ . The oxidation of cysteate is limited and entails less than 1 atom  $\text{O}_2$  uptake per mole.

By means of soluble extracts of the organisms we have been able



## SCHEME 4

PATHWAYS OF CYSTEINE SULFINATE METABOLISM IN *PROTEUS VULGARIS*

to map the metabolic routes by which cysteine sulfinat is degraded and have obtained evidence that the same reaction pattern is applicable to intact cells. There exists a dual pathway for the oxidation of L-cysteine sulfinat in this organism. One pathway is very rapid and commences with a transamination between the amino acid and  $\alpha$ -ketoglutarate or oxaloacetate, to produce L-glutamate or L-aspartate, and the keto acid analogue of L-cysteine sulfinat,  $\beta$ -sulfinylpyruvate.  $\alpha$ -Ketoglutarate is then regenerated by a TPN-specific glutamic dehydrogenase: the  $\alpha$ -ketoglutarate functions as a catalyst in the overall reaction.  $\beta$ -Sulfinylpyruvate, a close analogue of oxaloacetate, is readily desulfinated in the presence of  $\text{Mn}^{++}$ , similarly to the  $\beta$ -decarboxylation of oxaloacetate.

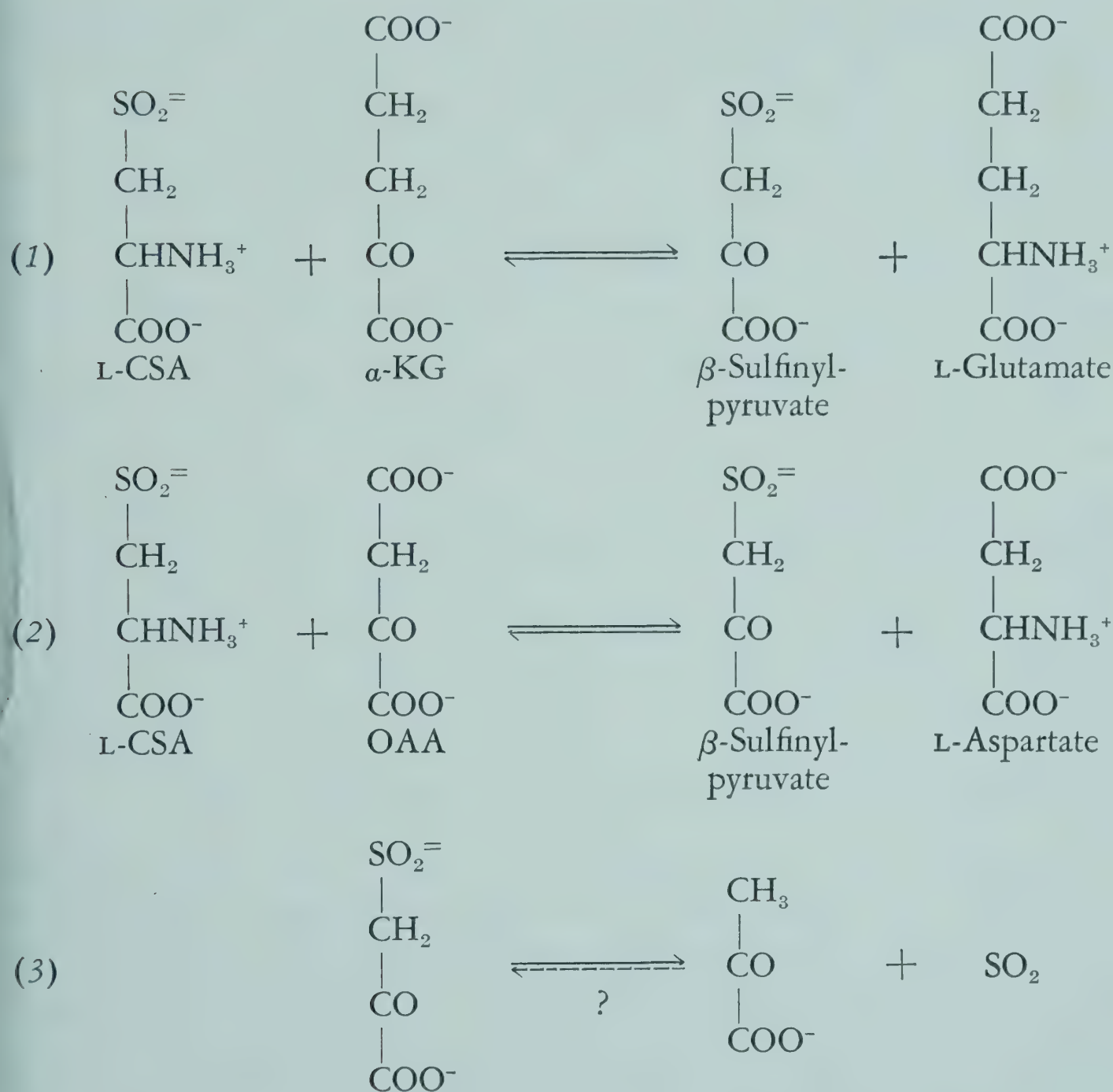
The sulfite arising in this reaction is rapidly and non-enzymatically oxidized to sulfate in the presence of a catalytic amount of  $\text{Mn}^{++}$ , but enzymatic oxidation of sulfite to sulfate has also been observed in the extracts in the absence of added  $\text{Mn}^{++}$  and with versene present. The latter reaction is very slow<sup>3</sup> compared with the  $\text{Mn}^{++}$ -catalysis and, unlike the sulfite oxidase of rat liver, it requires a dye, such as methylene blue, for electron transport (58).

The second pathway involves a reversible dehydrogenation of

<sup>3</sup> However, the rate is higher than that obtained by methylene blue alone, in the absence of added enzyme.

L-cysteine sulfinic acid to L-cysteate. The cysteate formed may transaminate with  $\alpha$ -ketoglutarate to yield  $\beta$ -sulfonylpyruvate ( $\beta$ -sulfo-pyruvate), a reaction previously demonstrated by Cohen (63).

## SCHEME 5

 CYSTEINE SULFINATE METABOLISM IN *PROTEUS VULGARIS*


The two transaminations which initiate the major pathway of cysteine sulfinic acid metabolism in *Proteus* (Scheme 5) have been studied in some detail. The outstanding feature of these reactions is their very high velocity. Even in crude extracts the turnover in the  $\alpha$ -ketoglutaric-cysteine-sulfinic reaction exceeds the rate of any other known transamination reaction (57). Secondly, with excess



$\alpha$ -ketoglutarate (or oxaloacetate) present and with added  $Mn^{++}$  to assure continuous removal of  $\beta$ -sulfinylpyruvate, the reaction goes to completion. With limiting  $\alpha$ -ketoglutarate and excess cysteine sulfinic acid, on the other hand, the reaction comes to an apparent equilibrium, despite continuous removal of one of the products. This observation will be amplified later on in this discussion.

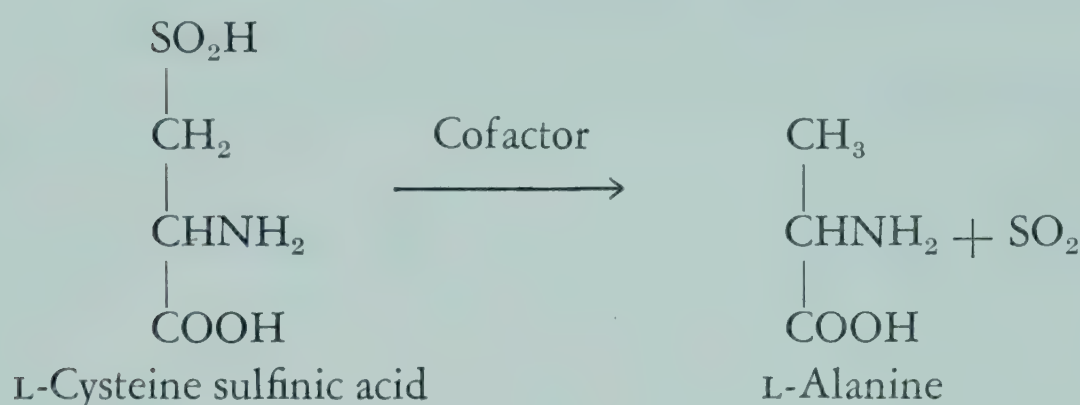
The question whether the desulfination of  $\beta$ -sulfinylpyruvate (Scheme 5) is an enzymatic process in the absence of *added*  $Mn^{++}$  remains unanswered. If enzymes exist for this reaction, the interesting possibility that a reversal of their action may provide a mechanism for the incorporation of inorganic sulfur into organic linkages, by analogy with the Wood-Werkman reaction, has been considered (Kearney and Singer, 57, 61) but not yet tested experimentally (cf. Addendum).

As regards the dehydrogenation of cysteine sulfinic acid to cysteate, this reaction is a relatively slow one, and the activity of the enzyme varies considerably in different preparations; in fact, it is absent in extracts prepared from cells grown under slightly different conditions from those used in the original experiments (64). The reaction requires a pyridine nucleotide coenzyme, which we have not been able to replace with DPN and TPN plus other cofactors, as judged by cysteic acid formation. The possible nature of the coenzyme for this reaction has been recently discussed elsewhere (65).

*Animal Tissues.* At the time our investigation on cysteine sulfinic acid metabolism was undertaken, Fromageot, Chatagner, and Bergeret (56, 60, 66, 67) had already described two overall reactions of this amino acid in rats in vivo and in extracts of rabbit liver: the decarboxylation of L-cysteine sulfinic acid to hypotaurine and the anaerobic desulfination of this amino acid to alanine and  $SO_2$  (Scheme 6). The latter reaction was of particular interest in that it required an unknown, thermostable cofactor. Shortly after the discovery of the transaminations of cysteine sulfinic acid in *Proteus*, Fromageot and associates (68) identified the cofactor of the desulfinase reaction as  $\alpha$ -ketoglutarate and postulated a double transamination, involving the  $\alpha$ -ketoglutarate-glutamate couple, as the probable route of

## SCHEME 6

## THE DESULFINASE REACTION OF RABBIT LIVER



*Probable mechanism:*

- (1) L-CSA +  $\alpha$ -KG  $\rightarrow$   $\beta$ -sulfinylpyruvate + L-glutamate
- (2)  $\beta$ -sulfinylpyruvate  $\rightarrow$  SO<sub>2</sub> + pyruvate
- (3) pyruvate + L-glutamate  $\rightarrow$  L-alanine +  $\alpha$ -KG

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SUM: L-CSA  $\rightarrow$  L-alanine + SO<sub>2</sub>

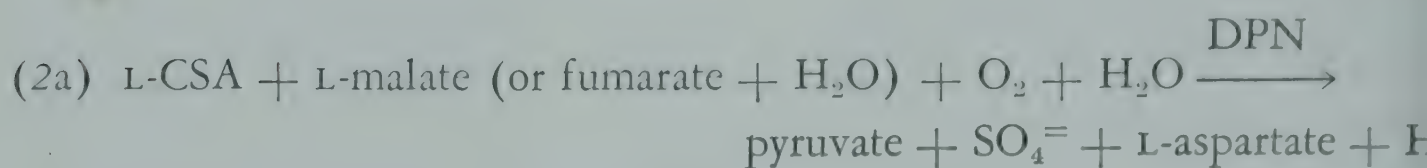
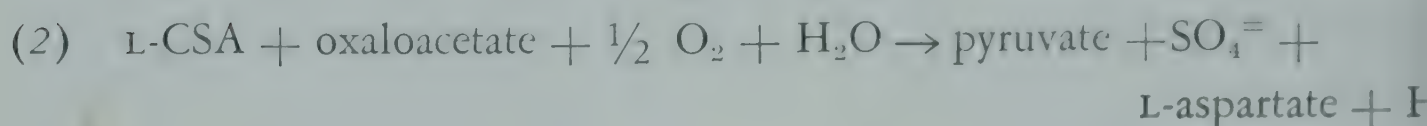
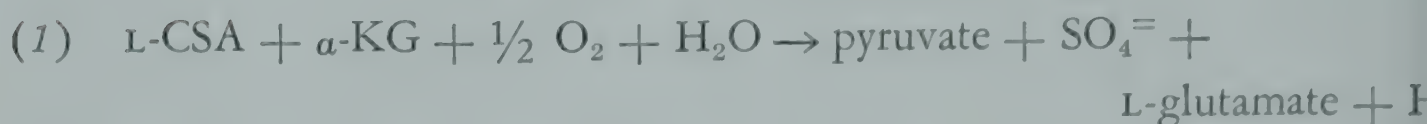
alanine formation in their preparations. This observation suggested that the main pathway of cysteine sulfinic acid metabolism in *Proteus* might also be operative in animal tissues and prompted us to undertake a systematic investigation of the enzymatic reactions of L-cysteine sulfinic acid in animal tissues. These studies were concentrated on mitochondrial preparations (acetone powders of mitochondria (69) and soluble extracts thereof) wherein most of the metabolic activity toward L-cysteine sulfinic acid is located. While the reactions to be described have been encountered in a wide variety of preparations of mitochondrial origin from various species, the majority of our studies have utilized rat liver and beef heart as the source of enzymes.

In all cases studied, the rate of cysteine sulfinic acid metabolism was very high and, with the exception of one reaction, the oxidation of this amino acid was initiated by a transamination with either  $\alpha$ -ketoglutarate or oxaloacetate, as in *Proteus*. The major overall conversions of cysteine sulfinic acid in rat liver mitochondrial extracts are summarized in Scheme 7.

The rapidity and completeness of the transamination of cysteine sulfinic acid with oxaloacetate makes possible the efficient oxidation of



## SCHEME 7

COUPLED REACTIONS OF L-CYSTEINE SULFINATE IN RAT LIVER  
MITOCHONDRIAL EXTRACTS

precursors of oxaloacetate, such as fumarate and L-malate (Scheme 7 reaction 2a), which alone are not appreciably oxidized by these preparations because of equilibrium considerations. Since the endogenous respiration of these preparations is nil, there is very little utilization of cysteine sulfinat without an added  $\alpha$ -keto acid (or precursor of same) and the keto acid is not removed at all in the absence of cysteine sulfinat. When both cysteine sulfinat and a source of oxaloacetate are added, the utilization of both substrates is stoichiometric, as illustrated in Table 1 (58).

It may be noted in Table 1, exp. 1, that the  $\text{O}_2$  uptake and  $\text{SO}_4^{=+}$  formation are somewhat less than required by the stoichiometry of

TABLE 1  
BALANCE OF L-CYSTEINE SULFINATE (L-CSA) METABOLISM IN RAT LIVER  
MITOCHONDRIAL EXTRACTS

Additions $\mu\text{M.}$	$\text{O}_2$ uptake * $\mu$ atoms	Removed $\mu\text{M.}$	Aspartate formed $\mu\text{M.}$	Glutamate formed $\mu\text{M.}$	Pyruvate formed $\mu\text{M.}$	$\text{NH}_3$ formed $\mu\text{M.}$	$\text{SO}_4^{=+}$ formed $\mu\text{M.}$
10 Fumarate + 15 L-CSA	16.5	10.2	9.1	0	9.9	0	Ca.
10 $\alpha$ -KG + 15 L-CSA	4.6	—	0	4.4	4.2	0	Ca.

\* In the presence of 1 mg. methylene blue the oxidation of sulfite to sulfate by the system is incomplete, giving rise to only 0.6-0.8 atoms  $\text{O}_2$  uptake per mole.

the reaction. The reason for this is that in the presence of methylene blue, which is used here as an electron transport dye between DPNH diaphorase and  $O_2$  for full operation of the malic dehydrogenase, the oxidation of sulfite to sulfate is incomplete.

A second point in this Table that should be mentioned is that while the oxaloacetate–cysteine-sulfinic acid reaction goes essentially to completion, as judged by the figures for aspartate and pyruvate formation, the corresponding transamination with  $\alpha$ -ketoglutarate fails to go to completion, despite the fact that  $\beta$ -sulfinylpyruvate is cleaved in the system as fast as it is formed. Further study of this point revealed that in transaminases from *Proteus* as well as from animal tissues, the transamination of oxaloacetate with cysteine sulfinic acid is complete, whichever member is limiting, if  $\beta$ -sulfinylpyruvate is removed from the system; but that with all preparations tested the  $\alpha$ -ketoglutarate–cysteine-sulfinic acid reaction comes to a halt before 50 per cent transamination is reached, *whenever* the keto acid is present in limiting amounts, despite the fact that on thermodynamic grounds the reaction is expected to go to completion. This paradoxical behavior was first called to our attention by Dr. Grisolia, who has also shown that the  $\alpha$ -ketoglutaric–aspartic reaction similarly comes to an apparent equilibrium, despite continuous rapid removal of oxaloacetate (70).

In connection with these transaminations of cysteine sulfinic acid it should be mentioned that the classical  $\alpha$ -ketoglutaric–aspartic transaminase, as prepared by the method of Cammarata and Cohen (71), at the highest stage of purification catalyzes the transamination of L-cysteine sulfinic acid with  $\alpha$ -ketoglutarate much faster than the corresponding reaction with L-aspartate (58). Experiments designed to decide whether both of the above transaminations of cysteine sulfinic acid are catalyzed by the same enzyme,  $\alpha$ -ketoglutaric–aspartic transaminase, are now in progress.

$\beta$ -Sulfinylpyruvate, the primary product of transaminations involving L-cysteine sulfinic acid, does not accumulate in the mitochondrial extracts, even in the presence of versene to bind traces of  $Mn^{++}$ . Instead, pyruvate and sulfite accumulate in beef heart mitochondrial



extracts, whereas in rat liver preparations pyruvate and sulfate are the only demonstrable products.

The question whether  $\text{SO}_4^{2-}$  arises in animal tissues from  $\beta$ -sulfinylpyruvate by oxidation of the latter to  $\beta$ -sulfonylpyruvate and cleavage to sulfate, or by desulfination followed by oxidation of the sulfite to sulfate, has been answered as follows. An analytically pure sample of  $\beta$ -sulfonylpyruvate (kindly provided by Dr. A. Meister) was not cleaved to pyruvate by any of the enzyme preparations from animal tissues or *Proteus*, nor was the accumulation of this compound observed in reactions (1) and (2) of Scheme 7. This rules out  $\beta$ -sulfonylpyruvate as an intermediate in the oxidation of L-cysteine sulfinic acid and implicates cleavage of  $\beta$ -sulfinylpyruvate to  $\text{SO}_2$  and pyruvate as the reaction mechanism, as in *Proteus* (57) and rabbit liver (68). Furthermore, the characteristics and extent of the  $\text{O}_2$  uptake in reactions (1) and (2a) of Scheme 7 parallel exactly those of the enzymatic oxidation of sulfite in liver preparations (58).

Time permits only a very brief summary of present knowledge of the last step in cysteine sulfinic acid metabolism, the oxidation of sulfite to sulfate. Heimberg, Fridovich, and Handler (72) have reported that this oxidation in partially purified preparations of whole rat liver ( $Q_{\text{O}_2} = 44$ ) has a pH optimum of 9.3, shows a pronounced induction period, and is inhibited by versene (73). More recent studies in Handler's laboratory (74) seem to suggest the requirement for two enzymes and for inorganic sulfate in the oxidation. In our laboratory sulfite oxidase was studied in extracts of rat liver mitochondrial acetone powder, which have a higher  $Q_{\text{O}_2}$  than previously reported preparations from whole rat liver ( $Q_{\text{O}_2} = 60$ ); they show a broad pH activity curve for sulfite oxidation with a maximum at 7.3; the preparation is not inhibited by versene and does not exhibit a lag period (58). Despite these apparent differences we may be dealing with the same enzyme. The resolution of these discrepant observations lies clearly in further purification of the oxidase.

In addition to the above reactions, a non-transaminative type of oxidation of cysteine sulfinic acid has also been observed in rat liver

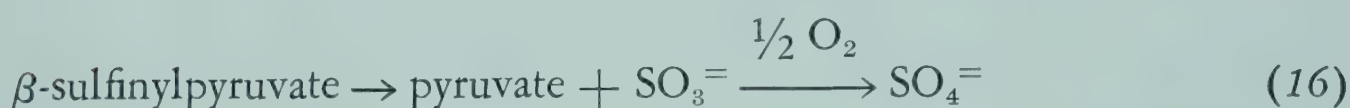
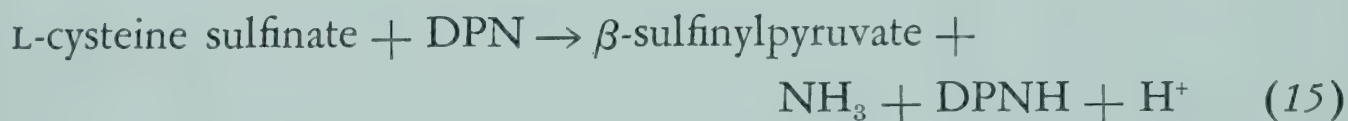
mitochondrial acetone powders (58). The reaction requires only DPN and an autooxidizable dye (diaphorase is present in the preparation). The reaction products are pyruvate,  $\text{SO}_4^{=}$ , and  $\text{NH}_3$ ; no amino acid accumulates in the reaction (paper chromatography). The reaction has been observed only in rat liver mitochondria; it is relatively slow compared with the transaminations discussed, and the activity of the enzyme shows considerable variation in different preparations. The simplest interpretation of its stoichiometry (Table

TABLE 2  
DEHYDROGENATION OF L-CYSTEINE SULFINATE IN RAT LIVER  
MITOCHONDRIAL EXTRACTS

Substrate $\mu\text{M.}$	$\text{O}_2$ uptake $\mu$ atoms	CSA removed $\mu\text{M.}$	Pyruvate formed $\mu\text{M.}$	$\text{NH}_3$ formed $\mu\text{M.}$	Aspartate or glutamate formed
15 L-CSA	12.6	15	12.5	12.3	0



2) is that it is an anaerobic pyridine nucleotide dehydrogenase, analogous to glutamic dehydrogenase, and that the reaction sequence involved is:



Only traces of cysteic acid were detected when L-cysteine sulfinat e was oxidized by rat liver mitochondrial acetone powder, and no hypotaurine accumulated under the experimental conditions.

The results presented indicate that cysteine sulfinat e is a highly active metabolite in animal tissues, as in microorganisms, and that the main end products of its aerobic metabolism are the same as those of cysteine: pyruvate and inorganic sulfate. These facts strengthen the view that cysteine sulfinat e is on the main pathway



of oxidation of cysteine. Furthermore, the results confirm Pirie's hypothesis of 20 years ago, that inorganic sulfate in animal tissues arises from cysteine sulfinic acid (53). There remains a consideration of the origin of taurine.

*The formation of taurine.* Current concepts of the interrelations of cysteine sulfinic acid, cysteate, and taurine are summarized in Scheme 8. The slow decarboxylation of L-cysteic acid to taurine in extracts of dog liver was demonstrated by Blaschko (75). The decarboxylation of L-cysteine sulfinic acid to hypotaurine by an analogous, slow reaction was first shown by Fromageot and associates (56, 60, 67) and was recently confirmed by Awapara (76). It is entirely possible that both decarboxylations are catalyzed by the same enzyme.

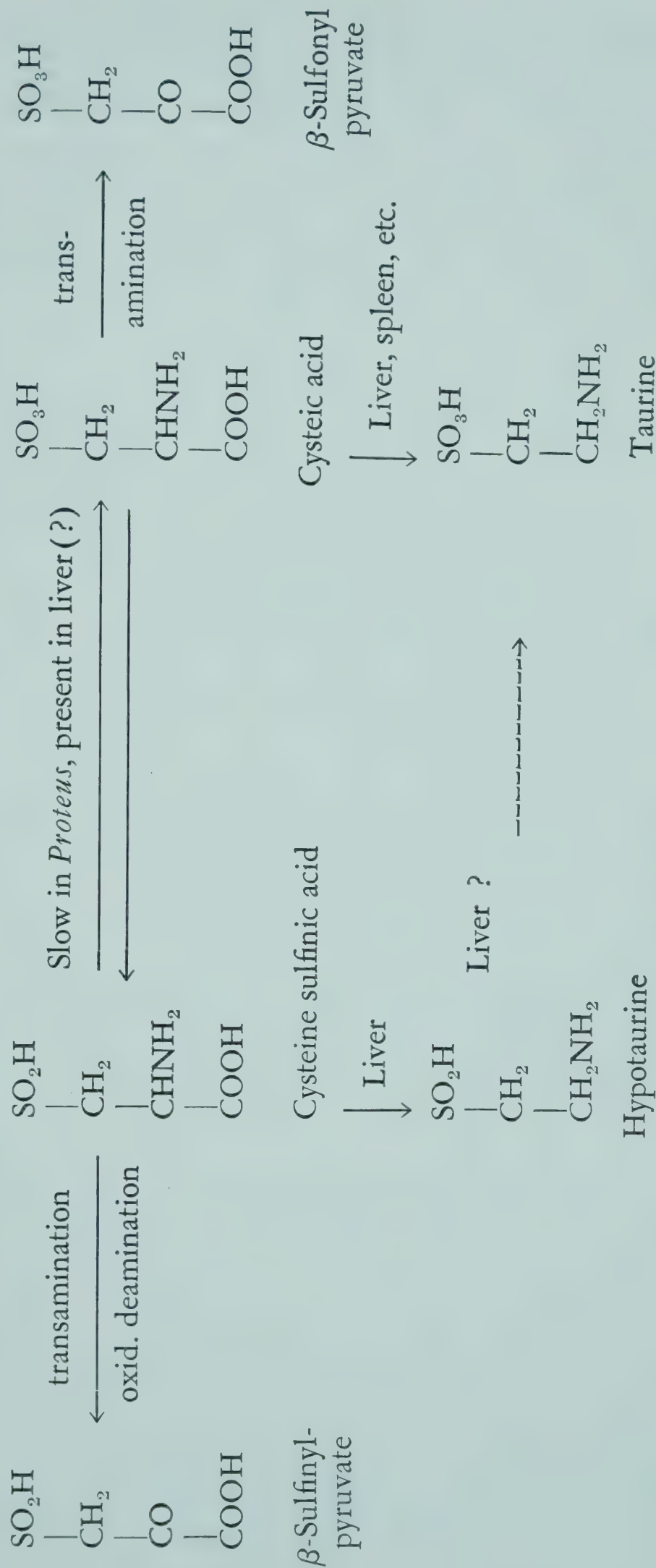
Although experiments in vivo (59) on the conversion of massive injected doses of L-cysteic acid to taurine confirm the view that cysteic acid is a potential precursor of taurine, from other experiments in vivo on the rates of conversion of  $S^{35}$ -labeled cysteine to hypotaurine, taurine, and cysteic acid, Awapara and Wingo (59) have come to the conclusion that an alternate route of taurine formation exists, which involves decarboxylation of cysteine sulfinic acid and oxidation of hypotaurine to taurine.

#### THE COUPLED OXIDATION OF SUCCINATE AND CYSTEINE SULFINIC ACID

The last subject we should like to present concerns another, rather interesting instance of a coupled reaction of cysteine sulfinic acid, the elucidation of which has led to some unexpected results. Last year we reported (65, 78) that mitochondrial acetone powders prepared from a variety of mammalian and avian tissues catalyze the rapid oxidation of L-cysteine sulfinic acid, without the formation of cysteate, in the presence of a pyridine nucleotide preparation from yeast and a suitable dye. The same reaction occurs in ultrasonic extracts of *Proteus vulgaris* OX-19 which have been clarified by ultracentrifugation. The reaction may be followed manometrically by the measurement of  $O_2$  uptake. Under anaerobic conditions no reduction at 340  $m\mu$  is observed in the system. When the coenzyme preparation—

SCHEME 8

INTERRELATIONS OF CYSTEINE SULFINIC ACID, CYSTEIC ACID, AND OF THEIR DECARBOXYLATION PRODUCTS





which is an essential component of the system—is passed through a column of Dowex-1-Cl, neither the effluent (which contains all the pyridine nucleotide initially present) nor any of the eluates could induce oxidation of cysteine sulfinic acid, but the effluent and a narrow fraction of 0.01 *N* HCl eluate *together* restored the maximal rate of O<sub>2</sub> uptake (Table 3). The pyridine nucleotide component in the effluent was quantitatively replaced by DPN, but not by TPN (77).

TABLE 3

RESOLUTION OF COMPONENTS OF COENZYME PREPARATION ON DOWEX-1-CL

Additions	Cu.mm.O <sub>2</sub> in 20 min.
Yeast preparation before Dowex chromatography	130
Dowex unadsorbed fraction	8
0.01 <i>M.</i> HCl eluate peak	17
Unadsorbed + eluate	139

Manometric assay in presence of phenazine methosulfate, L-cysteine sulfinic acid, and extract of rat liver mitochondrial acetone powder.

The factor in the eluate ("eluate factor") was assayed by measurement of the initial rate of O<sub>2</sub> uptake in the presence of excess DPN, cysteine sulfinic acid, phenazine methosulfate, and enzyme (Fig. 1). In routine work, extracts of beef heart, rat liver mitochondria, acetone powder, or extracts of *Proteus vulgaris* served as sources of the enzymes.

Considerable purification of the factor was achieved by chromatography of the pyridine nucleotide preparation on Dowex-1-Cl using increasing strength of HCl as eluant (gradient elution technique, 79). A typical experiment, in which the factor was assayed in the manometric test outlined, is shown in Fig. 2. The preparation thus obtained contained a solid dicarboxylic acid, besides some adenylic acid (5-AMP) as the only recognizable impurity; no P, S, N carbohydrate, or ultraviolet-absorbing material was present beyond that attributable to 5-AMP. The latter compound is not concerned with the reaction under study.

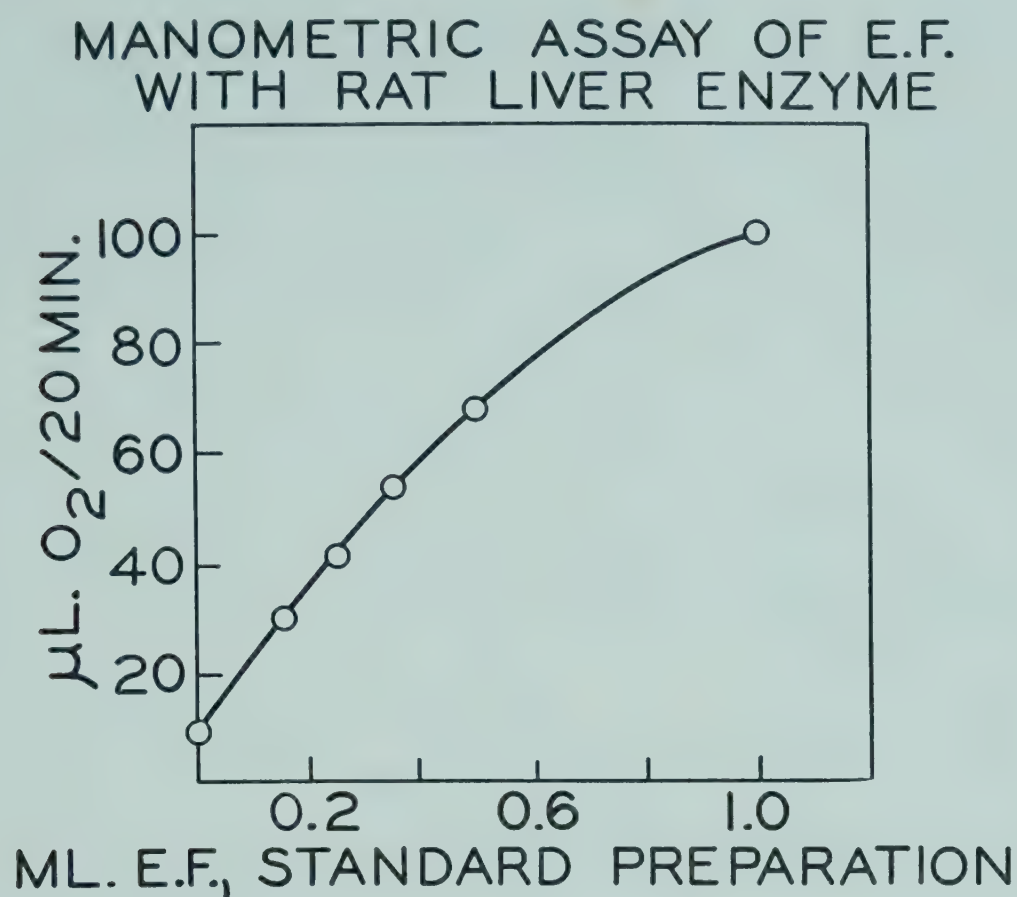


FIG. 1.

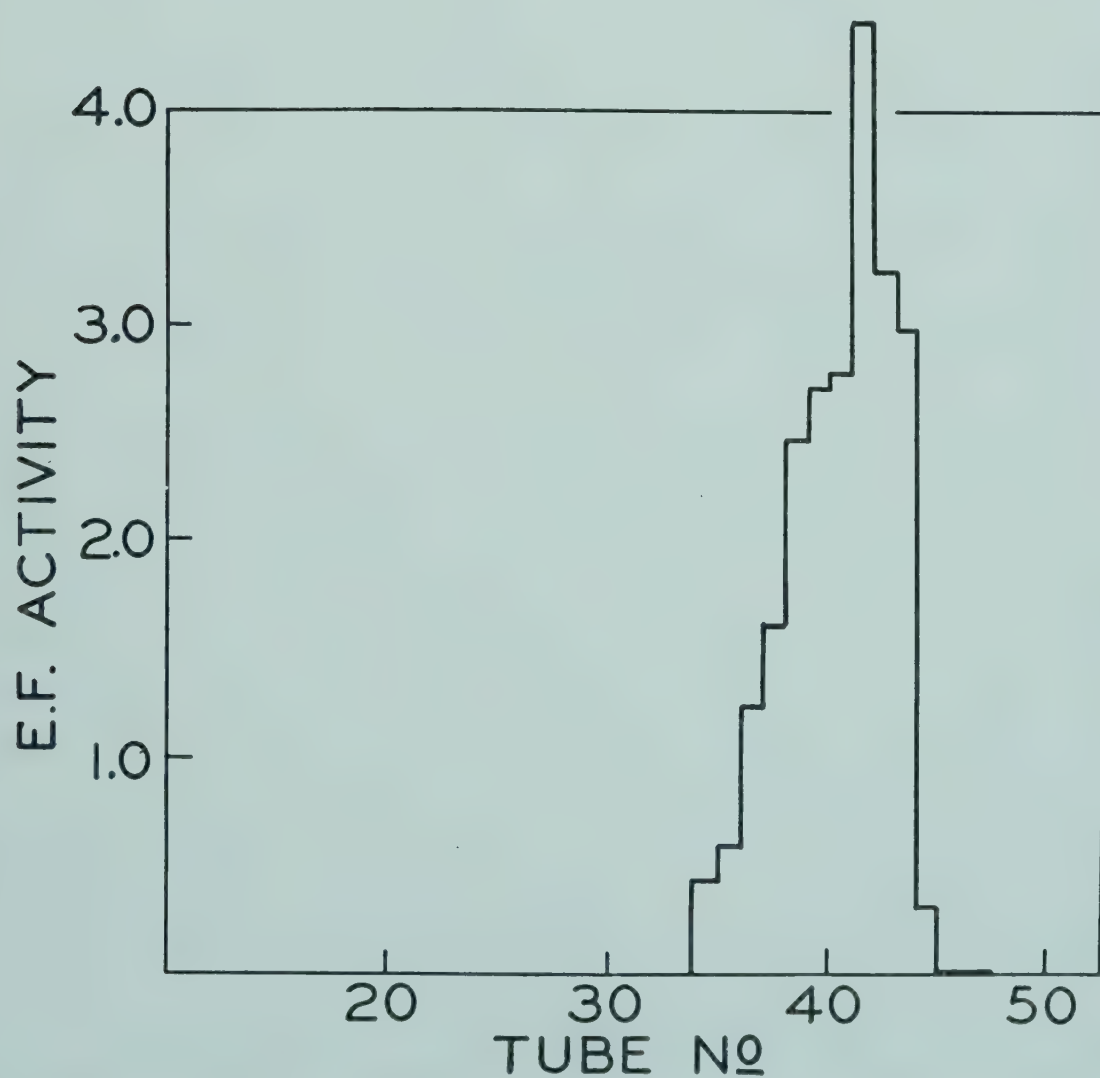


FIG. 2. Ion exchange chromatography of the "eluate factor" on Dowex-1-Cl. E. F. activity was measured by the manometric test shown in Fig. 1.



At this stage of our work it became apparent that the factor was not functioning as a catalyst, but rather as a substrate in the reaction. As seen in Fig. 3, in the presence of  $20\ \mu\text{M}$ . cysteine sulfinic acid the total  $\text{O}_2$  uptake at the completion of the reaction depended on the absolute amount of the factor added; indeed, there was a clean stoichiometric relation between total  $\text{O}_2$  uptake and amount of factor

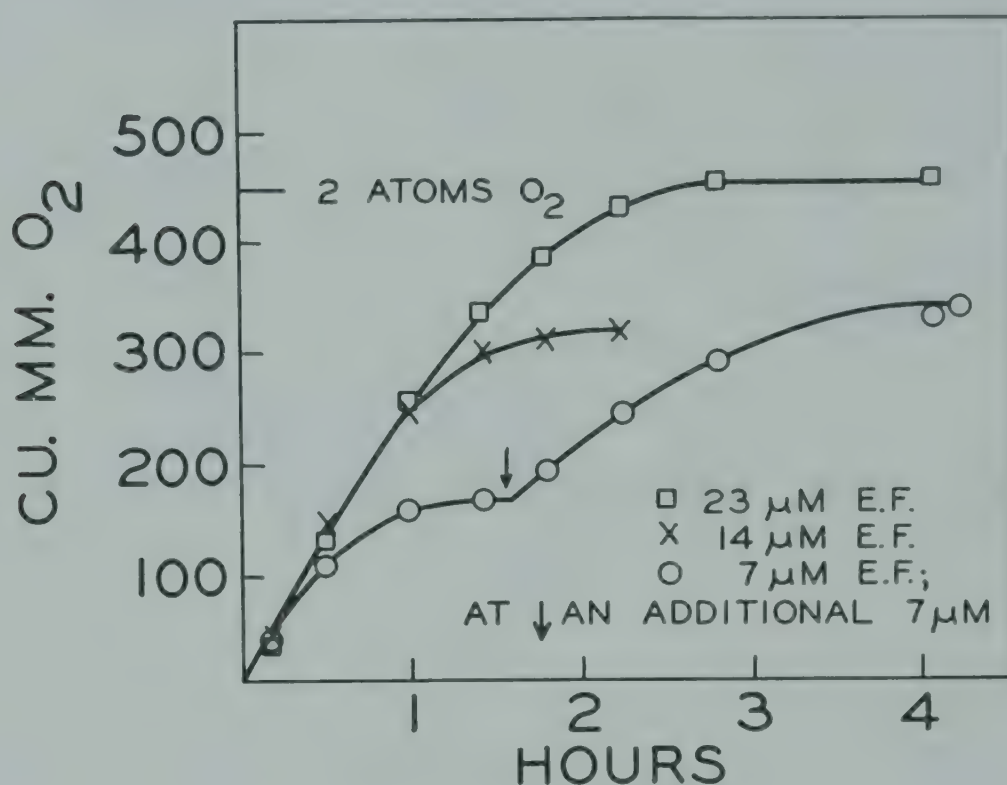


FIG. 3. The relation of *total*  $\text{O}_2$  uptake to "eluate factor" concentration. Manometric experiment with L-cysteine sulfinic acid, DPN, and methylene blue present in excess; a suspension of rat liver mitochondrial acetone powder in  $0.03\ \text{M}$ . phosphate, pH 7.3, was the source of the enzymes.

added. With excess cysteine sulfinic acid and DPN, the factor was rapidly and quantitatively utilized in a coupled reaction: one mole each of cysteine sulfinic acid and of the factor reacted to give one mole each of aspartate, pyruvate, and sulfate (77). In the absence of cysteine sulfinic acid the purified factor was only very slowly oxidized by the enzyme preparations in the presence of DPN, and faster in its absence. The products indicated (aspartate, pyruvate, and sulfate) could have arisen from the coupled oxidation of cysteine sulfinic acid and malate (or fumarate), in view of the reactions previously discussed, but the purified factor contained no malate or fumarate.

The question of the identity of the factor was eventually answered

by isolation. The purified preparation was treated with 5-nucleotidase to remove 5-AMP and was rechromatographed on Dowex-1-Cl. The fractions active in the enzyme assay were combined, taken to dryness, and twice recrystallized. The material was then readily identified as succinic acid (Table 4). Succinic acid quantitatively

TABLE 4  
IDENTIFICATION OF ELUATE FACTOR

Compound	M. P.	Mixed m. p.	Neutral equivalent	Solvent A	R <sub>F</sub> Solvent B	pK' <sub>1</sub>
Eluate factor	190°	190.5°	58.6	0.68	0.89	4.2
Succinic acid	190.5°		59	0.69	0.90	4.19

replaced the factor in the coupled oxidation in all of the enzyme preparations tested (Table 5). The balance of reaction products is in satisfactory agreement with the formulation:

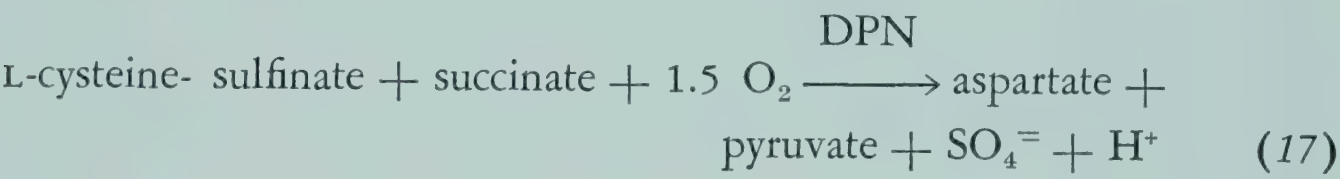
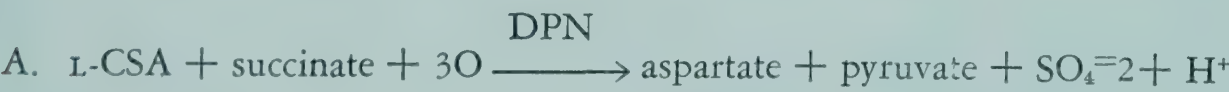


TABLE 5  
STOICHIOMETRY OF COUPLED OXIDATION IN LIVER PREPARATIONS

Substrate μM.	O <sub>2</sub> uptake * μ atom	CSA removed μM.	Aspartate formed μM.	Pyruvate formed μM.	SO <sub>4</sub> <sup>=</sup> formed μM.
A 10 succinate + 15 CSA	27.8	9.8	10.1	9.7	0.7-0.8
B 10 fumarate + 15 CSA	16.0	10.2	9.1	9.4	0.6

\* In presence of 1 mg. methylene blue the oxidation of sulfite to sulfate is incomplete (0.6-0.8 atoms O<sub>2</sub>/mole).





The following observations are offered as evidence that the reaction sequence is succinate  $\rightarrow$  fumarate  $\rightarrow$  malate  $\xrightarrow{\text{DPN}}$  oxaloacetate; oxaloacetate + cysteine sulfinic acid  $\rightarrow$  aspartate +  $\beta$ -sulfinylpyruvate  $\rightarrow$  pyruvate +  $\text{SO}_3^{=}$   $\xrightarrow{\text{liver}}$   $\text{SO}_4^{=}$ . First, the coupled reaction is competitively inhibited by malonate. Second, the entire reaction sequence depends on the initial dehydrogenation of succinate, as shown by the following experiments. Homogenates of rat liver mitochondrial acetone powder catalyze both the overall coupled reaction (17) and the one-step oxidation of succinate to fumarate with either methylene blue or phenazine methosulfate as carrier. Soluble extracts from this source catalyze the coupled reaction (17) and the oxidation of succinate to fumarate in the presence of phenazine methosulfate, but neither reaction occurs with methylene blue as the carrier. Nonetheless, when fumarate is substituted in the above experiment with methylene blue present, fumarate + cysteine sulfinic acid are smoothly oxidized to the same products, but 1 atom less  $\text{O}_2$  is consumed than in the presence of succinate (Table 5, reaction B). This also shows that all of the enzymes in the postulated scheme are present in the extracts.

These observations clarified the role of succinate in the oxidation of cysteine sulfinic acid in the rat liver system, inasmuch as succinate generates the oxaloacetate needed for the transamination of cysteine sulfinic acid. There remained the question as to what role the amino acid played in the oxidation of succinate, since in the coupled reaction the oxidation of succinate proceeds more vigorously and much more extensively than in the absence of cysteine sulfinic acid, even when DPN is omitted and no oxaloacetate, a known inhibitor of succinic dehydrogenase (80), is formed. In order to answer these questions and in view of the fact that we had obtained succinate oxidation in a soluble system, we decided to study the succinic dehydrogenase activity of these preparations in greater detail.

If these preparations contain a typical succinic dehydrogenase, then it should be possible to demonstrate the stoichiometric formation of fumarate from succinate in a one-step reaction. This has

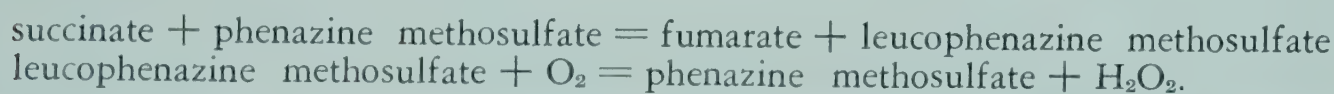
been accomplished (Table 6), using a partially purified preparation of the dehydrogenase derived from a soluble extract of beef heart mitochondrial acetone powder.

TABLE 6  
STOICHIOMETRY OF SUCCINATE OXIDATION

O <sub>2</sub> uptake μM.	Fumarate formed	
	theory *	found **
3.51	3.51	3.36

One-step reaction, succinate alone as substrate.

\* Calculated from the relation:



\*\* Determined, after deproteinization, with crystalline fumarase + malic dehydrogenase.

Fig. 4 compares the characteristics of the one-step oxidation of succinate to fumarate and the coupled oxidation of cysteine sulfinic acid + succinate to aspartate, pyruvate, and sulfite in extracts of heart mitochondria. In the latter case, besides succinate, excess DPN, cysteine sulfinic acid, crystalline fumarase, and highly purified malic dehydrogenase and transaminase were also added. Phenazine methosulfate was the dye in both experiments. It is apparent that in the coupled reaction the initial rate is maintained much longer than in the one-step reaction. The reason why the *initial* rate in the upper curve is approximately double is, of course, that the coupled reaction entails twice as great an O<sub>2</sub> uptake as the succinate → fumarate step. The relationship illustrated in these curves holds throughout the purification, and it is also observed in purified *particulate* preparations of the enzyme, prepared by the method of Green et al. (81).

The reactivity of various dyes with the dehydrogenase is summarized in Table 7. Suspensions of rat liver mitochondrial acetone powders can utilize methylene blue and phenazine methosulfate equally well. After centrifugation, the soluble enzyme is incapable of reaction with methylene blue, but all the activity with respect to the phenazine dye is retained. Thus the reaction of the primary



dehydrogenase with methylene blue requires at least one other factor, which is not extracted by this procedure. The same is true of the reaction with 2,6-dichlorophenol-indophenol and cytochrome *c*. All these dyes have been widely used in the past for the assay of

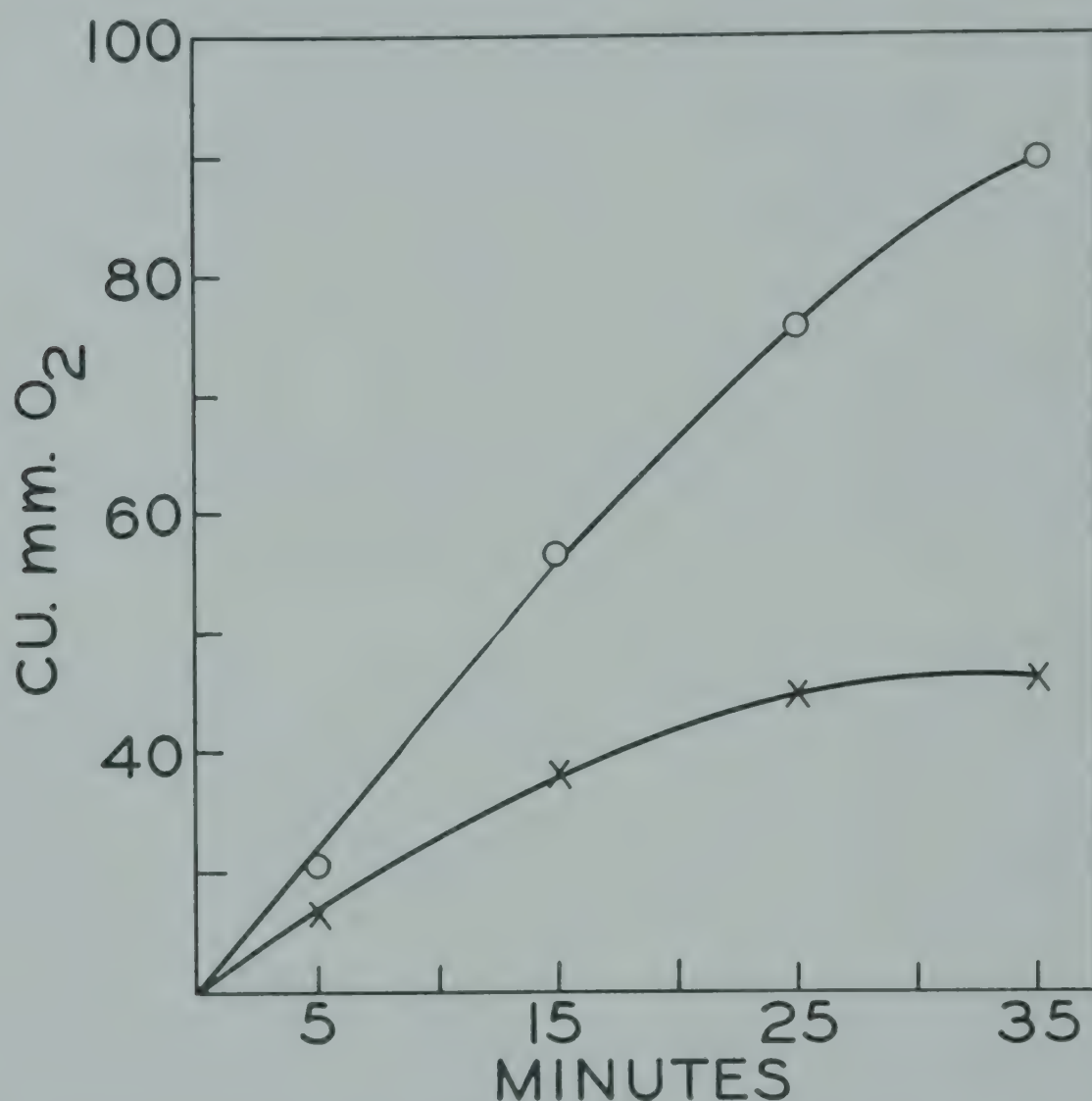


FIG. 4. Succinate oxidation by soluble, purified succinic dehydrogenase, isolated from beef heart mitochondria. Lower curve, 0.02 *M*. succinate, 0.05 *M*. phosphate, pH 7.6, and 1 mg. phenazine methosulfate present. Upper curve, same + 0.02 *M*. L-cysteine sulfinate,  $4 \times 10^{-4}$  *M*. DPN, and the following enzymes added in excess: crystalline fumarase, highly purified malic dehydrogenase and oxaloacetic-glutamic transaminase. (The latter enzyme catalyzes the oxaloacetic-cysteinesulfinic transamination.) Temperature, 38° C.

succinic dehydrogenase. In the coupled reaction ferricyanide is only about 40 per cent as effective as phenazine methosulfate; when succinate alone is used as substrate, only 5 per cent of the maximal rate is observed with ferricyanide. We believe that the low reaction rates with ferricyanide are at least partly due to the inhibition of the dehydrogenase by this —SH inhibitor.

TABLE 7

REACTIVITY OF DYES AND CYTOCHROME C WITH SUCCINIC DEHYDROGENASE

Enzyme preparation	Relative reaction rate				
	Phenazine methosulfate	Methylene blue	Ferri-cyanide	2,6-dichlorophenol-indophenol	Cyt. c
Rat liver mitochondrial acetone powder	100	100	—	—	—
Soluble extract of same	100	0	—	—	—
Beef heart mitochondrial acetone powder	100	0	43	—	—
Soluble extract of same	100	0	39	0	0
Soluble ultrasonic extract of <i>Proteus vulgaris</i>	100	37	—	—	0

These data indicate that of the dyes tested phenazine methosulfate is the only suitable one for the assay of the dehydrogenase, and they may, in part, explain previous apparent failures in obtaining the majority of the succinic dehydrogenase activity of tissues in soluble form.

TABLE 8

SOLUBILIZATION OF SUCCINIC DEHYDROGENASE

Enzyme preparation	Succinic dehydrogenase activity		
	Units/ml.	% extracted	Units/mg. protein
Homogenate of particulate enzyme * . . . .	94.4		4.7
pH 8.9 TRIS extract	84.4		32.4
Extract after 1 hr. centrifugation at 108,000 × g	90.8	96%	42.8

\* Beef heart mitochondria, frozen and thawed in KCl, soluble proteins discarded, extracted with ter-amyl alcohol, soluble fraction discarded, residue dehydrated with acetone.

Unit = μl. O<sub>2</sub> uptake per min.

The claim that this is a soluble preparation is based on the data presented in Table 8, which shows that essentially all the dehydrogenase activity of beef heart mitochondrial acetone powders is



extracted with dilute TRIS buffer, pH 8.9, and remains in solution after 1 hour at  $108,000 \times g$ , as well as on the fact that the activity remains in solution throughout the pH range of 4.6 to 9.0, and finally, on the criterion that it is readily fractionable by classical methods.

TABLE 9  
PURIFICATION OF SUCCINIC DEHYDROGENASE

Step	$Q_{O_2}$
Beef heart mitochondrial acetone powder	117
TRIS extract of same	600
Beef heart mitochondria, frozen-thawed, amyl-alcohol-treated residue, acetone powder	280
TRIS extract of same	1500
After 1st $(NH_4)_2SO_4$ fractionation	3150
After $Ca_3(PO_4)_2$ gel and 2nd $(NH_4)_2SO_4$ fractionation	6840

Table 9 summarizes the purification which has been attained so far. Typical preparations obtained by this procedure possess 60 times the  $Q_{O_2}$  of beef heart mitochondrial acetone powders on succinate. In practice, instead of using mitochondrial acetone powders as a source, we utilize a step elaborated by Green and associates (81) in their work on the particulate dehydrogenase. The mitochondria are twice frozen and thawed, with centrifugations in between, and the residue is extracted with 10 per cent tertiary amyl alcohol in order to remove proteins easily solubilized, prior to making the acetone powder.

The best preparations so far obtained are by no means homogeneous. However, the relative ease of purification of the enzyme, using the assay system described, offers a sound basis for the hope that the isolation of the pure dehydrogenase may not be too far off.

It may be pointed out that our purified preparations are free from hemin compounds. Thus cytochrome *b* is clearly not identical with succinic dehydrogenase. If cytochrome *b* plays an obligatory role in succinate oxidation, it must be a part of the electron-transport system.

The reason why in the coupled reaction with cysteine sulfinic acid, linearity and regular kinetics are maintained longer than in the straight dehydrogenation is not clear. It is conceivable that in soluble preparations and in purified particulate preparations provisions have to be made to "pull the reaction" by continuous removal of fumarate. The reasons may be in the energetics of the primary dehydrogenation step. The removal of fumarate by other means, such as fumarase + TPN + malic enzyme, or fumarase + malic dehydrogenase + glutamic-oxaloacetic transaminase + glutamate, also serve to maintain the initial rate to some extent, but they are far less efficient than the coupled reaction with cysteine sulfinic acid, in accord with the fact that the fastest and most complete removal of oxaloacetate is provided by its transamination with cysteine sulfinic acid.

It is also possible that the coupled reaction serves in some way to destroy the  $\text{H}_2\text{O}_2$  formed in the reoxidation of leucophenazine methosulfate, which would otherwise inactivate the dehydrogenase. At least in heart preparations the initial rate of the one-step reaction is maintained effectively by the addition of reducing agents or of sufficient amounts of catalase + ethanol.

#### ADDENDUM

In connection with Dr. Horowitz's remarks on the pathways of incorporation of inorganic sulfur compounds into amino acids, we should like to add a few comments. As we first pointed out two years ago, a likely major site of incorporation of inorganic sulfur into organic linkages in microorganisms is at the oxidation level of sulfite, and the reaction may involve the interaction of sulfite with pyruvate (or phosphopyruvate) to yield  $\beta$ -sulfinylpyruvate. The ubiquitous occurrence of very rapid transaminations of the latter compound provides a pathway for its conversion to cysteine sulfinic acid, which might then be reduced to cysteine. [The pathway cysteine sulfinic acid  $\rightarrow$  cysteate  $\rightarrow \text{SO}_4^{2-} \rightarrow \text{H}_2\text{S} \rightarrow$  cysteine appears unlikely at present, in view of the fact that the dehydrogenation of cysteine sulfinic acid to cysteate, where examined, was found to be either a very slow reaction or to be absent, and we have been unable to find



any evidence for the direct cleavage of cysteic acid (or of its keto analogue) to inorganic sulfate.]

This hypothesis does not negate the possible existence of a second mechanism of "sulfur fixation" which might involve  $H_2S$  as the primary reactant.

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# INTERMEDIATE STEPS IN THE BIOSYNTHESIS OF THREONINE

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EVIDENCE RECENTLY has accumulated that a primary precursor in the biosynthesis of threonine is L-aspartate. Conversion of the latter substance appears to occur through the intermediate steps outlined in Fig. 1. This scheme is supported by experiments (1) with micro-

## INTERMEDIATE STEPS IN THE BIOSYNTHESIS OF THREONINE

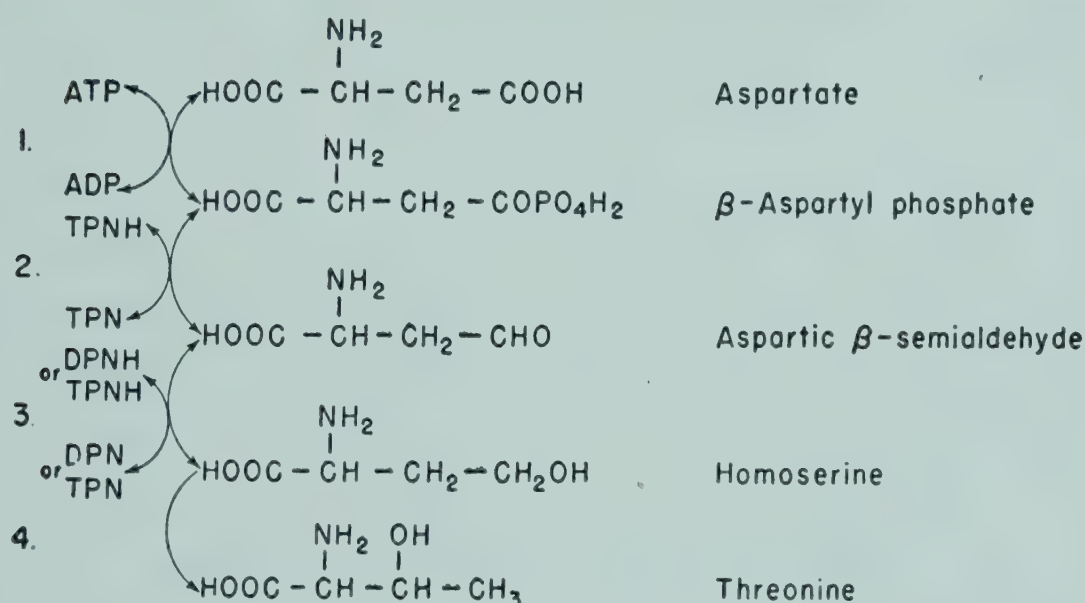


FIG. 1.

organisms using isotope-labeled substrates, (2) with threonineless mutants of several species, and (3) with isolated enzyme systems from yeast and from several mutants of *Escherichia coli*.

*Isotope evidence.* Ehrensward and his collaborators (13, 15) noted that *Torulopsis utilis* or *E. coli* grown on  $\text{C}^{13}$ - $\text{C}^{14}$ -labeled acetate yielded threonine and aspartate having identical isotope distributions in their 4-carbon chains, and suggested a biosynthetic relation between the two amino acids. Abelson et al. (1-2) made a similar



deduction from their observations that added aspartate or homoserine suppressed incorporation of  $C^{14}O_2$  and  $C^{14}$ -glucose into threonine in *E. coli*. More direct evidence was obtained by Delluva, who found that when 3- $C^{14}$ -aspartate was added to the growth medium of *E. coli*, threonine subsequently isolated was most heavily labeled in the 3-position (14).

*Genetic and nutritional evidence.* Working with washed suspensions of a mutant of *E. coli*, Hirsch and Cohen (19) found a substantial conversion of L-aspartate to L-homoserine. The latter accumulated because of this mutant's inability to convert it to threonine. Suspensions of another threonineless mutant, as well as of a wild-type strain, were shown by these workers to utilize L-homoserine (but not D-homoserine) for threonine synthesis (11).

The first evidence for the role of homoserine in threonine formation was the observation of Teas et al. (30) that this compound could substitute for threonine (as well as for methionine) in the nutrition of a mutant of *Neurospora*. The implication that it was a natural precursor was strengthened when they found that L-homoserine accumulated in other mutants of *Neurospora* (16).

Threonine was found to have a sparing effect on the aspartic acid requirement of several lactobacilli by Ravel et al. (28), a relation suggesting that threonine arises from aspartate in these organisms also.

#### EVIDENCE FROM YEAST ENZYMES

It has been found in our laboratory that  $C^{14}$ -labeled aspartate<sup>1</sup> incubated with a cell-free extract of baker's yeast yields  $C^{14}$ -labeled threonine. The latter has been identified by paper chromatography in four solvent systems, and by its sensitivity to periodate. Fractionation and study of the enzymes responsible for the aspartate-threonine conversion have revealed two new intermediate substances,  $\beta$ -L-aspartyl phosphate (BAP) and L-aspartic  $\beta$ -semialdehyde (ASA). These compounds accumulate in certain enzyme systems (5-8). They have also been prepared by chemical syntheses. Each of the new

<sup>1</sup> We wish to thank Dr. C. B. Anfinsen for a sample of  $C^{14}$ -labeled aspartic acid.

compounds undergoes two enzymatic reactions, as indicated in Fig. 1. The enzymes which catalyze these reactions (Steps 1-3) have been purified so that each is free of the others. Some properties of BAP and ASA and of the enzymes involved in their metabolism are discussed below.

*$\beta$ -L-Aspartyl phosphate (BAP).* BAP was synthesized (8) from carbobenzoxy-L-aspartyl- $\alpha$ -benzyl ester  $\beta$ -chloride (3). By shaking

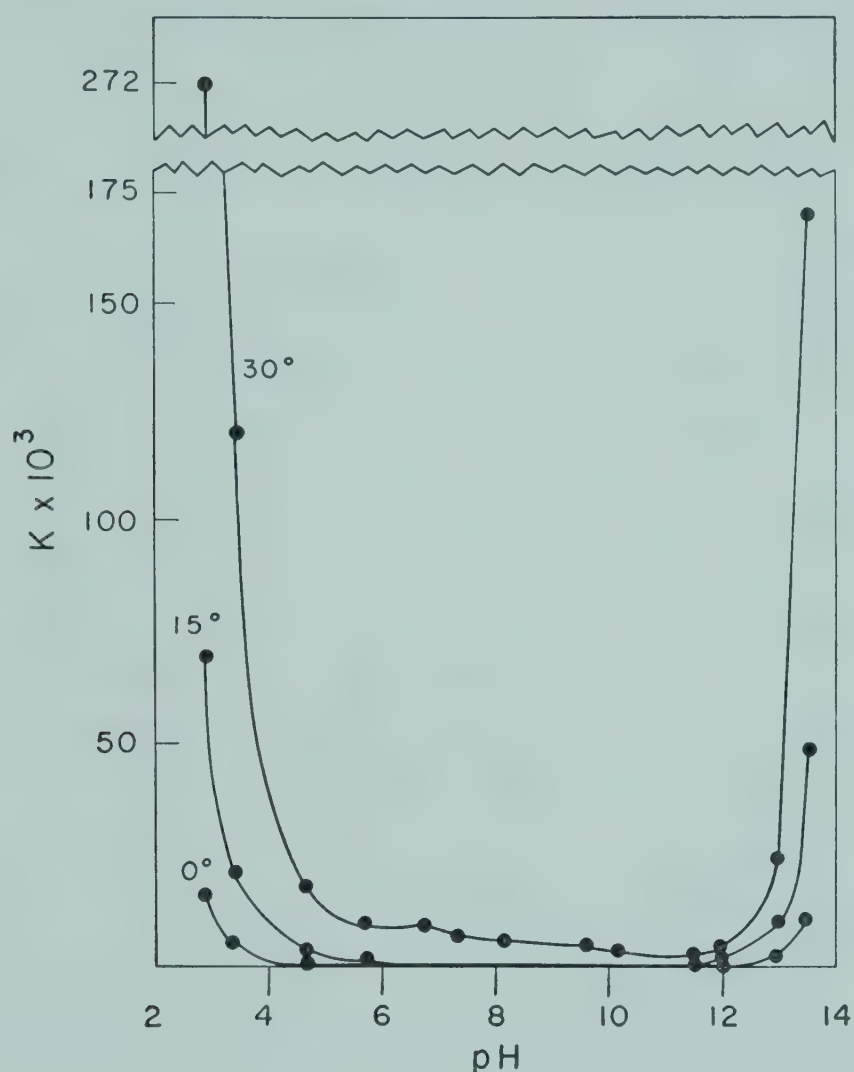


FIG. 2. First-order hydrolysis rates of  $\beta$ -L-aspartyl phosphate (BAP) as a function of pH at three temperatures (8).

in ether with monosilver phosphate (23) the chloride was replaced with phosphate. The carbobenzoxy and benzyl groups were then removed by hydrogenation over palladium black in cold potassium bicarbonate solution. The resulting solution of the potassium salt of BAP has been very useful in enzyme experiments. It may be stored for several weeks at  $-20^\circ\text{C}$ . Because of the compound's extreme lability, however, we have not been able to prepare it in a



solid state such as a silver, lithium, or barium salt. All attempts to purify it have led to excessive losses. It appears to be much more labile than the related acyl phosphates, acetyl phosphate (22) or 3-phosphoglyceryl phosphate (24). Succinyl monophosphate, however, is also quite unstable (20). Fig. 2 shows the rate of hydrolysis of BAP as a function of pH and temperature. At pH 7 and 30° C. the rate is three times the hydrolysis rate found by Koshland (21) with acetyl phosphate at pH 7 and 39° C. Though the hydrolysis data show greatest stability in mildly alkaline solutions, storage at low temperature is best in the neutral pH range.

*L-Aspartic  $\beta$ -semialdehyde (ASA)*. ASA hydrochloride was formed in quantitative yield (by enzymatic assay) when ozone was bubbled through a solution of allylglycine hydrochloride at 0° C. (8). It has been freed of other reaction products by adsorbing it on a column of Dowex-50 (hydrogen form), washing with a large volume of water, and eluting with 4N HCl. This compound is reasonably stable in acid solution but deteriorates markedly in a few hours in neutral solution. Very dilute neutral solutions (0.01 M. or less) are more stable than concentrated ones, suggesting that losses are due to polymerization. Evaporation to dryness of solutions of the hydrochloride, even at very low temperatures, causes destruction of much of the compound.

Allylglycine has been resolved by the method of Greenstein and collaborators (4)<sup>2</sup> into its D and L isomers, and the corresponding isomers of ASA prepared from them. Only the L-isomer is enzymatically active.

*$\beta$ -Aspartokinase (8)*. This enzyme catalyzes the phosphorylation of L-aspartate by ATP in the presence of  $Mg^{++}$  ions (Step 1). BAP accumulates in the reaction mixture until equilibrium is reached (Fig. 3). Addition of ADP, a reaction product, causes the concentration of BAP to fall to a new equilibrium level. ADP reacts with synthetic BAP in the same manner to yield ATP and L-aspartate.

<sup>2</sup> We are grateful to Drs. S. M. Birnbaum and J. P. Greenstein for hog kidney acylase, and for assistance with the asymmetric hydrolysis of acetyl DL-allylglycine.

The equilibrium constant for the reaction



is about  $3.5 \times 10^{-4}$ , a value very close to the constant found by Bücher for the analogous reaction between ATP and 3-phosphoglyceric acid (9). It is substantially lower, however, than the equilibrium constant for the acetokinase reaction, reported by Rose et al. to be about  $10^{-2}$  (29).

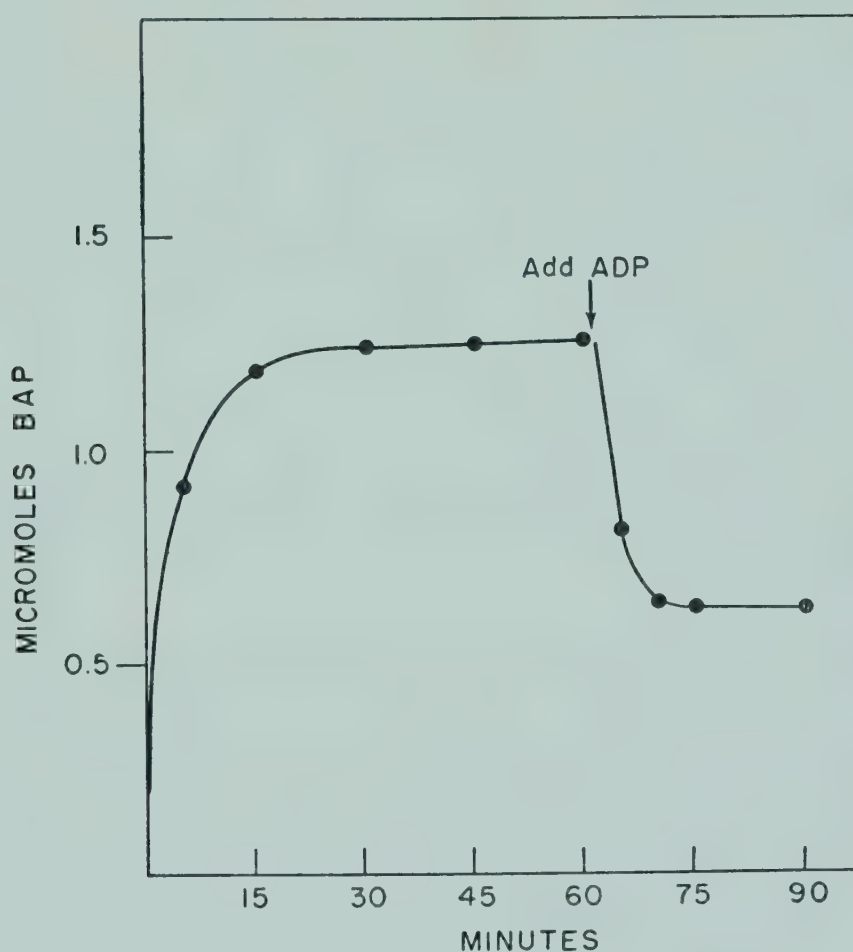


FIG. 3. Formation of  $\beta$ -L-aspartyl phosphate (BAP) in the  $\beta$ -aspartokinase reaction, and reversal by ADP (8). Initial concentrations of L-aspartate and ATP were 250 and  $21.3 \mu\text{M./ml.}$   $2.86 \mu\text{M./ADP per ml.}$  were added at the point indicated.

D-Aspartate and L-glutamate are not phosphorylated in this system.

$\beta$ -Aspartokinase is somewhat unusual in that it shows no marked point of optimum activity between pH 5 and 9.

*L-Aspartic  $\beta$ -semialdehyde dehydrogenase* (8). The reversible reduction of BAP to ASA catalyzed by this enzyme is illustrated in Table 1. At pH 7.0 BAP and TPNH disappear, and ASA forms in equivalent amounts. DPNH cannot substitute for TPNH. At pH

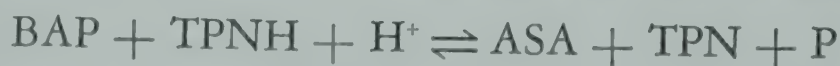


TABLE 1

INTERCONVERSION OF  $\beta$ -L-ASPARTYL PHOSPHATE (BAP) AND L-ASPARTIC  $\beta$ -SEMIALDEHYDE (ASA) BY ACTION OF ASA DEHYDROGENASE

	BAP $\mu M.$	TPNH $\mu M.$	ASA $\mu M.$
Forward reaction (BAP + TPNH added) pH 7.0	— 0.17	— 0.14	+ 0.14
As line 1 with DPNH for TPNH	— 0.02	—	0.00
Reverse reaction (ASA, TPN, and P added) pH 9.0	+ 0.16	+ 0.16	— 0.15

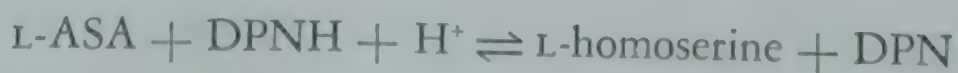
9.0 and with a relatively high concentration of phosphate the equilibrium favors BAP formation. ASA disappears with equivalent formation of BAP and TPNH according to the equation:



The equilibrium constant for this reaction is about  $3.0 \times 10^6$ . The corresponding value for the closely analogous phosphoglyceraldehyde dehydrogenase reaction is  $3.0 \times 10^7$ , according to information given by Bücher (9).

Participation of inorganic phosphate in the reverse reaction is shown by its effect on the reduction of TPN (Fig. 4). Arsenate may be substituted for phosphate to demonstrate TPN reduction. The enzyme also catalyzes an arsenolysis of BAP, just as 3-phosphoglyceraldehyde dehydrogenase causes an arsenolysis of acyl phosphate (17, 18, 27). Specificity for L-ASA and TPN are also shown in Fig. 4.

*L-Homoserine dehydrogenase* (8). In the presence of this enzyme L-homoserine, but not the D isomer, reduces DPN to DPNH (Fig. 5). Addition of acetaldehyde to the equilibrium mixture does not affect the DPNH level, showing that alcohol dehydrogenase is absent from the enzyme preparation. The quantities of L-homoserine and L-ASA added were, respectively, 10.0 and 0.05 micromoles, the ratio indicating that homoserine formation is greatly favored. The equilibrium constant for the reaction



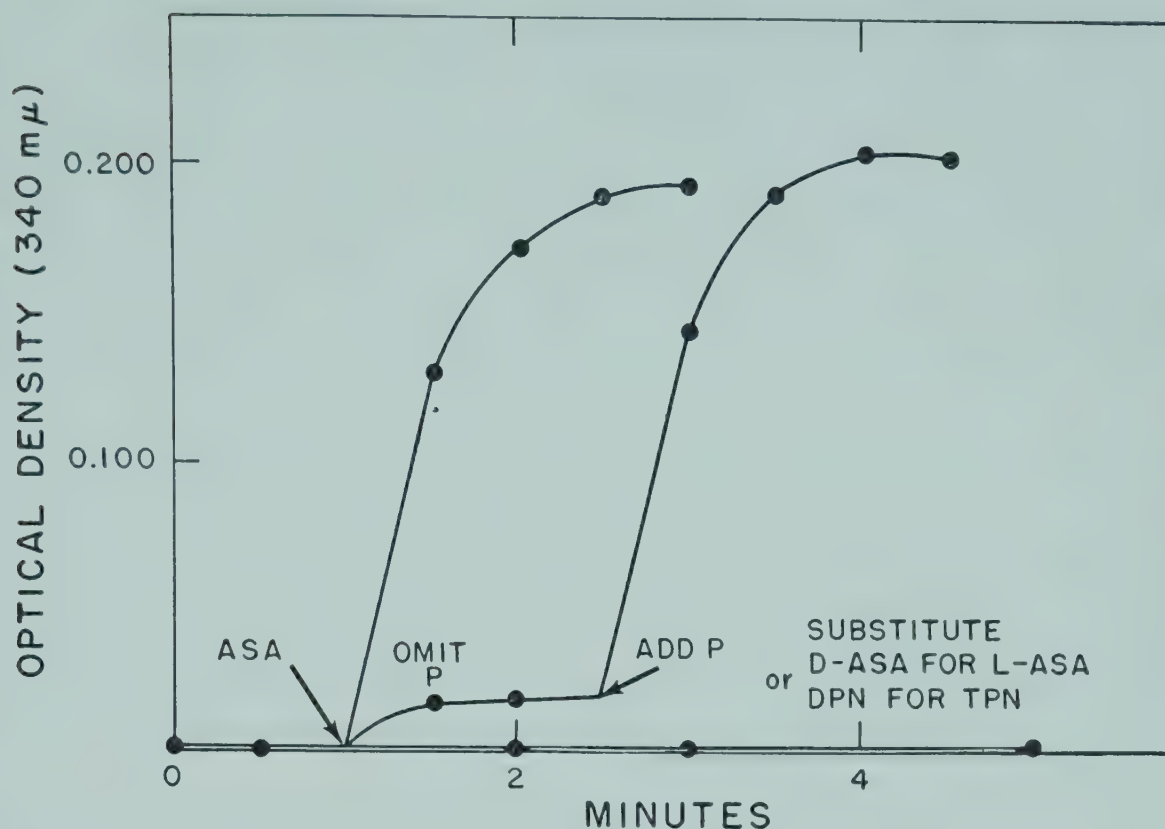


FIG. 4. Enzymatic reduction of TPN by L-aspartic  $\beta$ -semialdehyde (ASA), showing phosphate dependence and specificity.

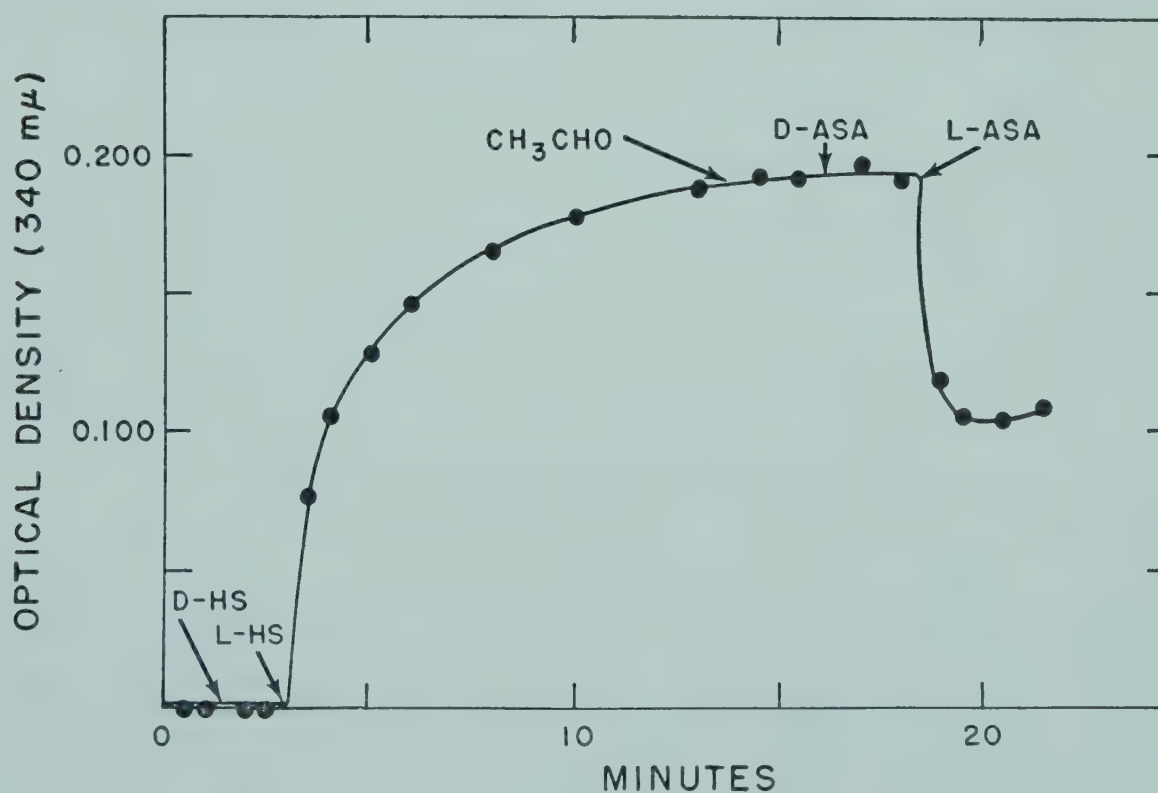


FIG. 5. Reduction of DPN by L-homoserine (L-HS), and reoxidation by L-aspartic  $\beta$ -semialdehyde (L-ASA) (8).



has been found to be about  $0.9 \times 10^{11}$ . This is very close to the value,  $1.07 \times 10^{11}$ , calculated from the data of Negelein and Wulff (25) for the alcohol dehydrogenase reaction. TPN reacts in the system also, but at about one-third the rate found with DPN.

This enzyme can be used to identify and quantitatively determine L-ASA. The amount of DPNH oxidized, determined spectrophotometrically, is equivalent to the L-ASA added to the system.

#### EVIDENCE FROM EXTRACTS OF *E. coli*

Recently G. N. Cohen, B. Nisman, and collaborators at the Pasteur Institute have obtained cell-free extracts of several mutants of *E. coli* in which the intermediate steps of threonine biosynthesis can be studied (10, 12, 26). Their findings are summarized below.

*Step 1.* L-Aspartate activation by ATP can be demonstrated in extracts of all mutants tested by formation of hydroxamic acid in the presence of hydroxylamine (26). Since coenzyme A can stimulate this activation (12), there is a possibility that  $\beta$ -aspartyl-CoA, rather than BAP, is the activated intermediate, or perhaps both occur.

*Steps 2 and 3.* An extract of one mutant which activates L-aspartate cannot reduce the latter. Extracts of a second mutant do reduce aspartate in a TPNH-dependent system but form no homoserine. A third mutant yields an extract which, with ATP and a source of TPNH, does form homoserine from L-aspartate (12). This series of experiments provides evidence for the reduction of an activated aspartate in two steps, such as steps 2 and 3 of Fig. 1.

*Step 4.* A substantial advance in the study of the conversion of homoserine to threonine was made when the French workers found this process to be dependent upon both pyridoxal phosphate and ATP. Though both substances are required for threonine formation, only ATP is necessary for homoserine disappearance (26).

Two types of evidence have been reported which indicate that at least one intermediate occurs between homoserine and threonine. (A) Homoserine in the presence of ATP and hydroxylamine appears

to form a hydroxamic acid, an indication of the existence of an activated intermediate (26). (B) Homoserine disappearance is faster than, and not parallel with threonine formation, as shown in Table 2. This suggests the accumulation of an intermediate in the early stages of the incubation and its later conversion to threonine.

TABLE 2

THREONINE FORMATION FROM HOMOSERINE IN AN EXTRACT OF *E. coli* IN THE PRESENCE OF ATP AND PYRIDOXAL PHOSPHATE (10).

Homoserine added $\mu M$ .	Incubation time hrs.	Homoserine utilized $\mu M$ .	Threonine formed $\mu M$ .
12.5	2	9.9	4.2
	4	10.3	6.5
25.0	2	14.7	4.3
	4	21.5	7.8
	12	24.0	15.0

Because their extracts contain a powerful threonine deaminase (which is partially inhibited by ATP)  $\alpha$ -ketobutyrate also accumulates in many of the French workers' experiments. They feel that the fact that threonine accumulates at all under these conditions may mean that the actual accumulated product is not threonine but an intermediate which forms threonine during their test procedure (10).

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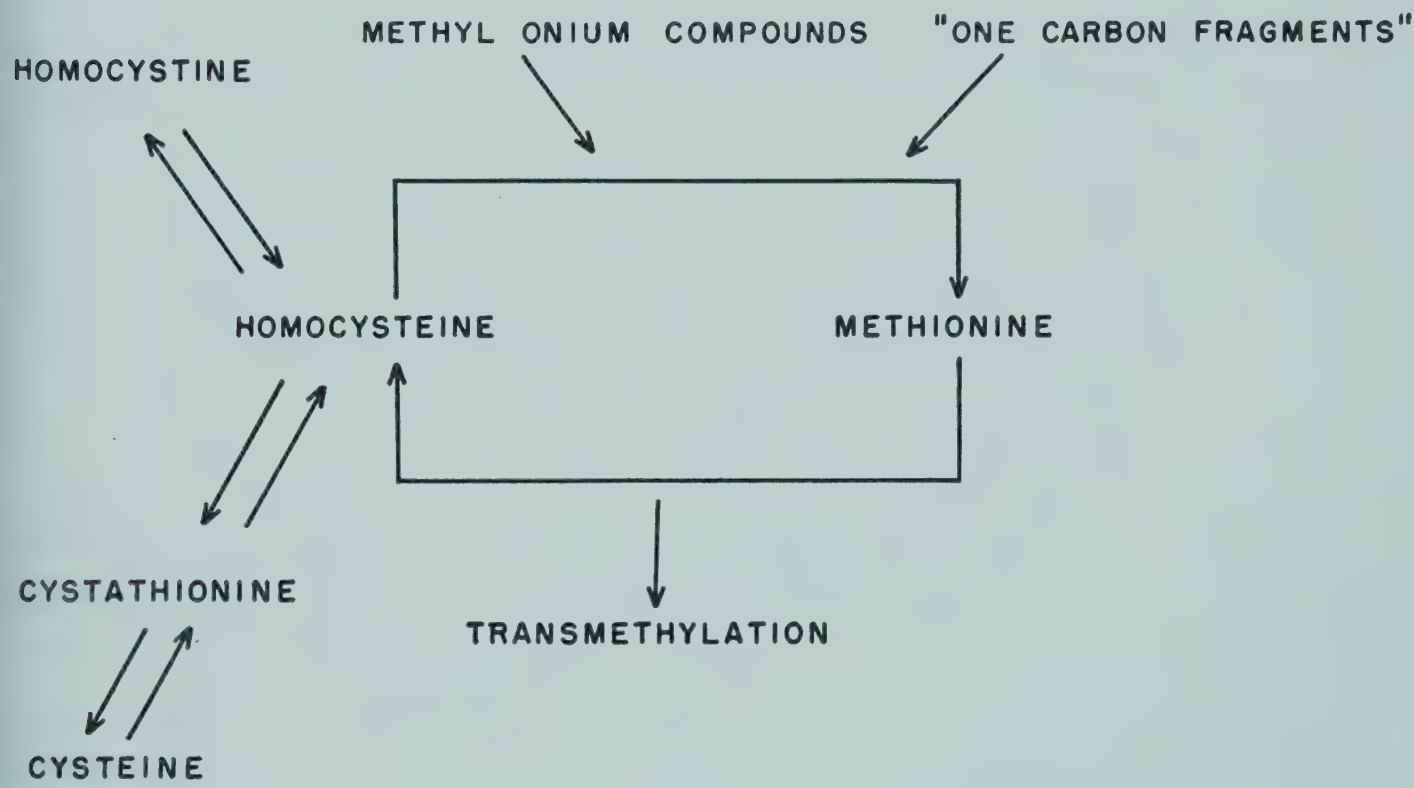
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# CONSIDERATIONS OF HOMOCYSTEINE AND ITS ROLE IN THE METABOLISM OF S-AMINO ACIDS \*

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IN THE TWENTY years since the discovery that homocysteine is formed by chemical decomposition of methionine (7), the role of homocysteine in intermediary amino acid metabolism, and more



Scheme 1.

particularly the relationship between homocysteine, methionine, and cysteine, have received a great deal of attention (Scheme 1). Homocysteine has been definitely implicated in at least three phases of the metabolism of S-amino acids: (a) in the synthesis of methionine,

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whether it be by transfer of the methyl group of onium compounds such as betaine or the thetins, or by synthesis *de novo* of the methyl group from one-carbon fragments; (b) in the metabolic interconversions known as transulfuration; and (c) in the transmethylation reactions in which methionine is the methyl donor, where logically, it has been presumed that homocysteine might be formed as a product. Recently, evidence for a more specific role of homocysteine (HSR) in the metabolism of one-carbon fragments, particularly in connection with the synthesis of purines and serine, has been advanced by my colleagues at Western Reserve University (5, 13, 8); and, no doubt, this aspect will be discussed by Doctors Sakami and Goldthwait in this symposium. In addition, homocysteine can undergo a reversible oxidation to the disulfide.

One peculiar feature of homocysteine is the fact that in spite of all the evidence pointing to its important metabolic function, so far as I know there is no conclusive evidence that this compound occurs naturally as a free amino acid, either in the sulfhydryl or in the disulfide form, or as a protein constituent. On the other hand, as is well known, a homocysteinyl radical is found in at least two naturally occurring amino acids: methionine and cystathionine.

The search for another compound containing the homocysteinyl radical was the logical extension of our work on enzymatic transmethylation reactions. As discussed here two years ago in some detail (8), there is good evidence that in a mechanistic sense methionine itself is not a methyl donor and that its enzymatic activation is a prerequisite to the transfer of its methyl group. The product of this activation reaction is S-adenosylmethionine (9), a sulfonium derivative of methionine and the adenosine moiety of ATP. The correctness of the proposed structure of S-adenosylmethionine has been confirmed recently by total synthesis (1). When tested enzymatically, the synthetic material was between 40 and 50 per cent as active as the natural material. The synthetic material was DL with respect to the  $\alpha$ -amino group and, presumably, it was also racemic at the sulfonium center. Therefore, the finding that the synthetic material is approximately 50 per cent as active as the



natural product is open to two interpretations. Experiments are under way, in cooperation with Doctors Baddiley and Jamieson of the Lister Institute, to clarify the relationship between stereo-configuration and biological activity. The suggestion that adenosylhomocysteine, rather than homocysteine, should be the primary product resulting from transmethylation reactions involving methionine follows directly from these findings (2, 17). This deduction has now been verified and confirmed experimentally by the identification of adenosylhomocysteine (ASR) (Fig. 1) as one of the

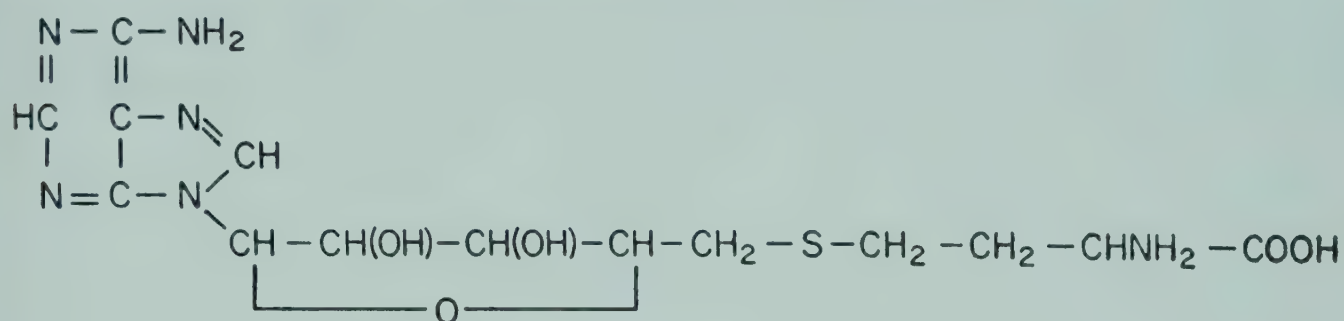
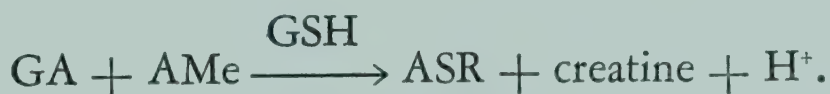


FIG. 1. The structural formula of S-adenosylhomocysteine.

products accumulating during the enzymatic synthesis of creatine (10). The reaction leading to the formation of adenosylhomocysteine is shown in the following equation.



In a typical experiment guanidinoacetic acid (GA), 120  $\mu\text{M}$ . and S-adenosylmethionine (AMe) 90  $\mu\text{M}$ ., labeled with  $\text{S}^{35}$  were incubated for 150 minutes with 70 units (80 mg.) of a partially purified preparation of GA methylpherase (11) in phosphate buffer (0.05  $\text{M}$ ., pH 7.4) and BAL (0.002  $\text{M}$ ). The incubation was terminated by addition of trichloroacetic acid. Unreacted AMe was removed from the protein filtrate by precipitation with ammonium reineckate. After removal of the excess reineckate, the supernatant fluid, containing S-adenosylhomocysteine (ASR), was made 0.02  $\text{N}$  with respect to  $\text{HCl}$  and passed through a small column of Norite A (500 mg.); the Norite was then washed with 100 ml. of water. The filtrate and washings which contained no radioactivity were discarded. The Norite column was then eluted with four 50 ml.



portions of aqueous pyridine (10%). The mobility of adenosylhomocysteine was followed by measuring the radioactivity of the eluates. The first two eluates, which contained over 90 per cent of the total counts, were pooled, freed from pyridine, and brought to a small volume under reduced pressure. This material, in addition to adenosylhomocysteine, contained creatine, guanidinoacetic acid, and traces of pyridine. For further purification, the Norite eluate was then applied in a thin band to a sheet of Whatman #1 paper and subjected to descending chromatography (solvent system ETOH, acetic acid, and water, 75:5:20). In this system creatine and guanidinoacetic acid separate readily from adenosylhomocysteine.

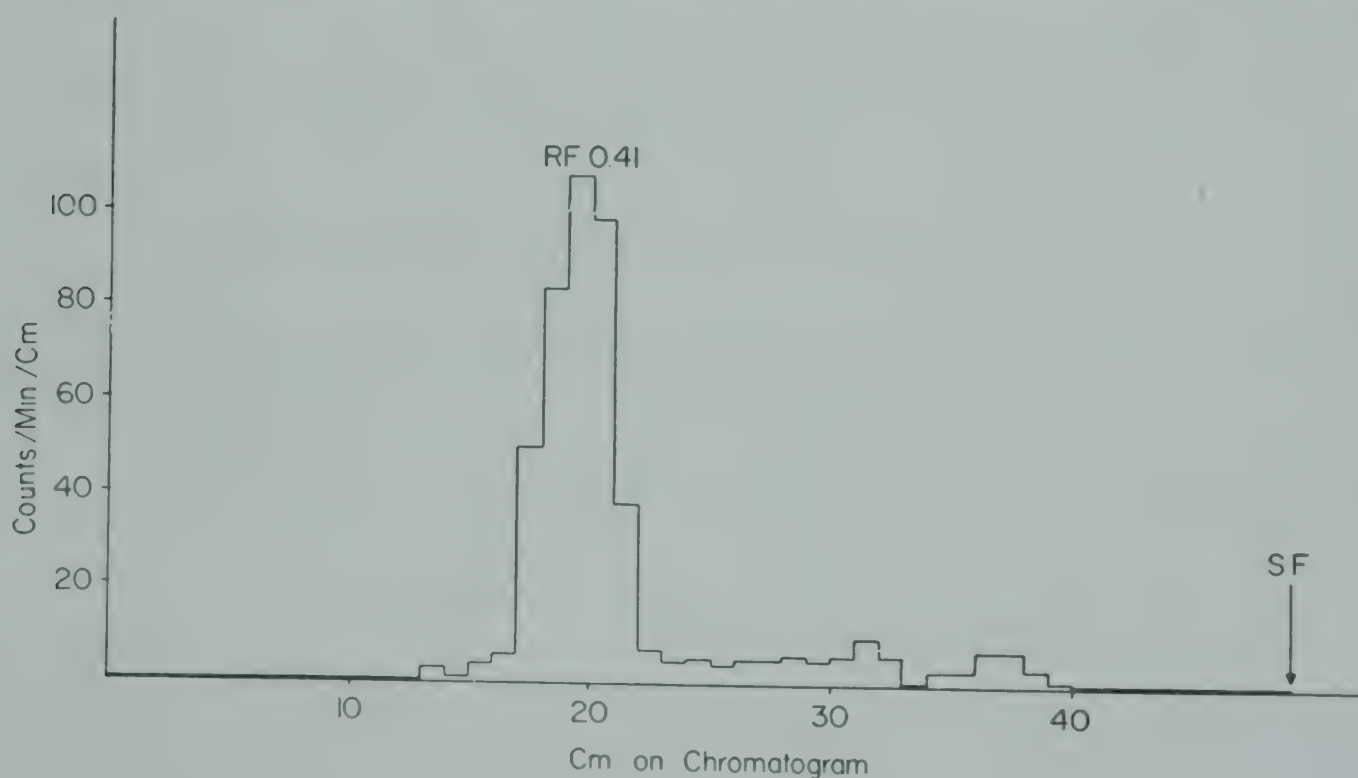
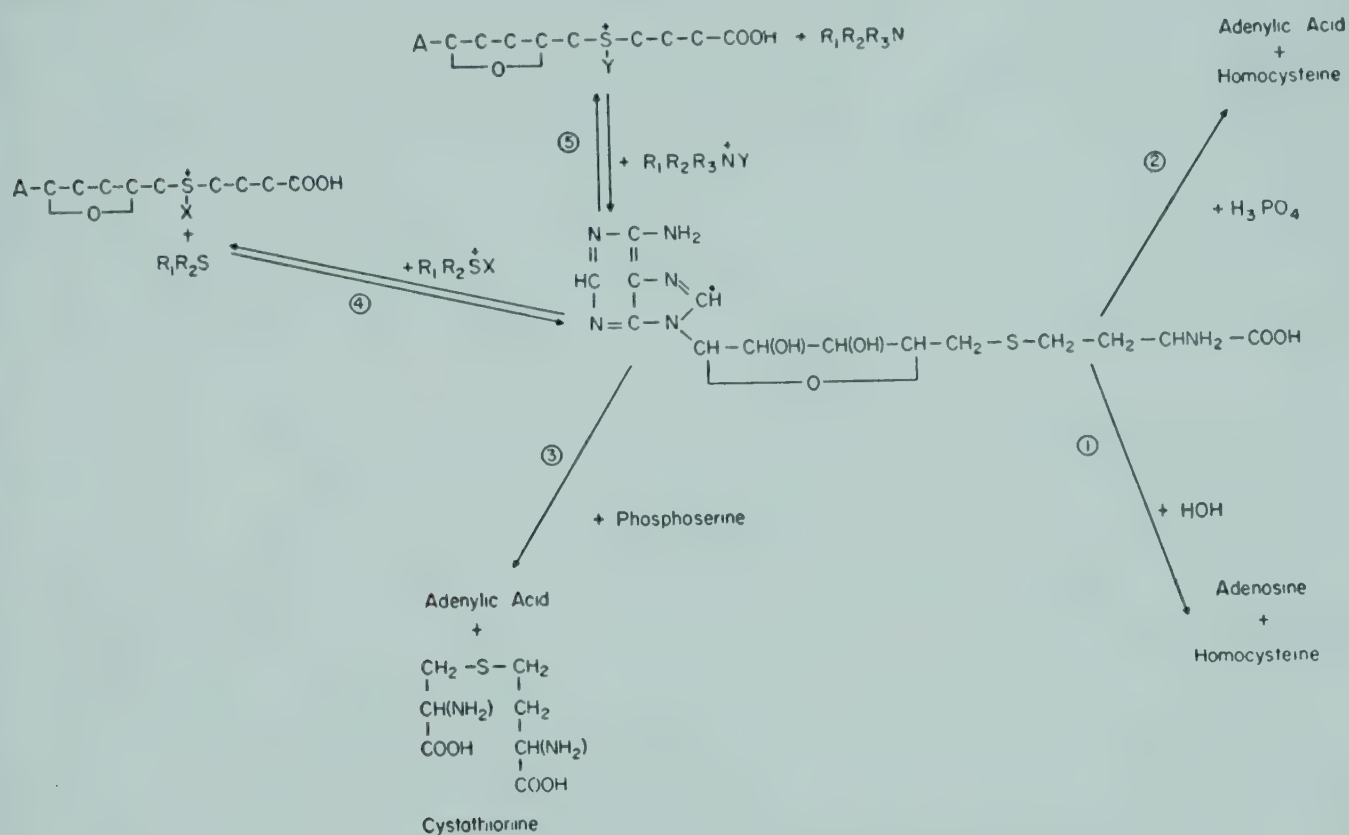


FIG. 2.

Examination of the chromatogram revealed one area exhibiting radioactivity, ultraviolet absorbency, and a ninhydrin-positive reaction (Fig. 2). This area was cut out, eluted with water, and the water eluate brought to small volume; on the basis of adenine content, 29  $\mu$ M. of purified ASR were obtained. The purified material behaved as a single substance when rechromatographed with several different solvents; it had an ultraviolet absorption spectrum characteristic for adenine nucleosides, with a maximum at 260  $m\mu$ .; for each mole of adenine it contained one mole of pentose; and it

gave a positive reaction with the ninhydrin test and with the nitroprusside test for a thio-ether. Conclusive proof that the product formed enzymatically is adenosylhomocysteine was provided by the finding that the purified material was identical to synthetic adenosylhomocysteine, which has been prepared by Baddiley and Jamieson in England (3), by a reaction of homocysteine in liquid ammonia with 2',3'-*o*-isopropylidene-5'-*o*-toluene-*p*-sulfonyl adenosine (4) (tosyl adenosine), at room temperature.



Scheme 2.

It might be interesting at this point to indulge in some speculation as to the possible further metabolic reactions in which adenosylhomocysteine may participate.<sup>1</sup> The reactions shown in Scheme 2 are entirely hypothetical, and so far no definite evidence has been obtained to indicate which ones, if any, occur; yet a symposium such as this is surely a place where one may indulge in the luxury of making wild speculations.

The first possibility that may be considered is that adenosylhomo-

<sup>1</sup> It is noteworthy that all three of the S-containing nucleosides so far discovered (AMe, ASR, and adenine-thiomethylpentose) are resistant to enzymatic deamination by the specific intestinal adenosine deaminase and by the less specific adenosine deaminase of takadiastase.



cysteine might act as a methyl acceptor, with the result that adenosylmethionine would be regenerated. As methyl donors in this case one would wish to consider the thetins, betaines, and other methyl onium compounds. Transmethylation reactions of this type could be expected to be reversible, unless coupled with reactions in which the energy of the onium bond is dissipated. Furthermore, the possibility that adenosylhomocysteine might participate in the metabolism of one-carbon fragments other than  $\text{CH}_3$  must be entertained, particularly in view of the evidence involving homocysteine in trans formylation reactions. The recent work of Flaks and Buchanan (12), Greenberg (14), Kisliuk and Sakami (15), and others (16) indicates quite clearly the participation of a coenzyme and other cofactors in the transmethylation reactions involved in the synthesis of inosinic acid from carboxamide ribotide and in the synthesis of serine.

The exact nature of the coenzyme or its mechanism of action is not entirely clear. The evidence points to a formylated and partially reduced derivative of tetrahydrofolic acid. It is clearly possible to visualize a derivative in which the formyl group is attached to an onium pole in the pterine ring of folic acid. Such a compound, if it exists, which is indeed hypothetical, might interact with adenosylhomocysteine to give a formyl or hydroxymethyl analogue of active methionine. This possibility has been suggested before, but no experimental evidence has yet been brought to bear on this matter. Alternatively, adenosylhomocysteine might function as the primary acceptor of the "one-carbon" fragment which would then become "activated" in the same manner as the methyl group of active methionine is activated for transmethylation reactions.

Next we might discuss briefly the possibility that adenosylhomocysteine might be involved in cysteine synthesis, perhaps by participating as a carrier of the homocysteinyl radical in the reaction, or reactions, leading to the formation of cystathionine, a known intermediate in transulfuration reactions. It should be pointed out, however, that there is some experimental evidence (5, 6) indicating that in the biosynthesis of cystathionine the substrates might be homo-

cysteine and serine *as such*, rather than the derivatives adenosylhomocysteine or phosphoserine, as suggested here (Scheme 2). Yet, on paper at least, this last mechanism is attractive to me, and might be considered as an alternative pathway in the biosynthesis of cystathionine.

Finally, we should consider the possibility that adenosylhomocysteine might undergo hydrolysis or phosphorolysis to yield free homocysteine, and adenosine or adenylic acid, respectively. Reactions of this type would provide a mechanism for the regeneration of homocysteine. Such a mechanism probably is necessary, since free homocysteine, rather than adenosylhomocysteine, may participate in some of the reactions we have been discussing, as well as in others—for instance, the reactions resulting in the synthesis of methionine by transmethylation, which have received only the briefest mention in this presentation.

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# ON THE FORMATION OF C<sub>1</sub> FRAGMENTS FROM SERINE \*

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THE BIOSYNTHETIC reactions of serine are exemplified in part by two kinds of cleavage. In one, it is decarboxylated to ethanolamine (1, 2), or utilized with loss of carboxyl for sphingosine synthesis (3); in the other, it dissociates to glycine (4) and a C<sub>1</sub> fragment (5). This fragment is very effectively converted into the methyl groups of choline (1, 2) as well as the methyl of thymine (6) and C-2 and C-8 of purines (5). The following discussion is limited to several aspects of this conversion.

## THE STABILITY OF HYDROGEN IN THE $\beta$ -POSITION OF SERINE

Several years ago Elwyn and Weissbach, in our laboratory, found that when L-serine labeled with both C<sup>14</sup> and D in the  $\beta$ -position was administered to rats, and the incorporation of isotope into the methyl groups of choline was measured, the ratio of C<sup>14</sup>:D was substantially unaltered (7). These and other results are shown in Table 1. For comparison, an experiment with DC<sup>14</sup>OONa was performed, and the equal utilization of D and C<sup>14</sup> of formate for methyl groups (8) was confirmed.

Similar results were obtained by Elwyn in the synthesis of the methyl group of thymine (9) (Table 2). The higher deuterium dilutions in the latter may be due, in part, to the availability of only small amounts of pyrimidine for analysis.

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TABLE 1  
UTILIZATION OF L-SERINE-3-C<sup>14</sup>,D AND  
DC<sup>14</sup>OONa FOR CHOLINE METHYL GROUPS

Compound fed	Dilution		Ratio of dilutions (C <sup>14</sup> /D)
	C <sup>14</sup>	D	
Serine I A *	33	36	0.92
B	45	50	0.90
C	126	120	1.05
Serine II	69	54 †	1.3
Serine III	125	137	0.91
DC <sup>14</sup> OONa	53	50	1.06

\* L-Serine I was prepared by reduction of HC<sup>14</sup>(OH) = C(NHCOPh)—CO<sub>2</sub>Et with D<sub>2</sub>; L-Serine II, by reduction of DC<sup>14</sup>(OH) = C(NHCOPh)—CO<sub>2</sub>Et with H<sub>2</sub>; L-Serine III, by condensation of CD<sub>2</sub> with ethyl acetamidomalonate (5). The significance of the various methods for introducing deuterium into the serine molecule is discussed below.

† Deuterium value in methyl groups was only 0.024 atom per cent excess due to inadvertently low D concentration in serine.

TABLE 2  
UTILIZATION OF L-SERINE-3-C<sup>14</sup>,D AND  
DC<sup>14</sup>OONa FOR THYMINE METHYL GROUPS (9)

Compound fed *	Dilution		Ratio of dilutions (C <sup>14</sup> /D)
	C <sup>14</sup>	D	
Serine I A	44	60	0.73
B	43	65	0.66
C	46	51	0.90
Serine III	116	142	0.82
DC <sup>14</sup> OONa	65	73	0.89

\* See first foot-note to Table 1 for methods of synthesis of the labeled serine.

Abundant evidence is available that folic acid is involved in the metabolism of C<sub>1</sub> fragments which are characterized by a lower level of oxidation than that of CO<sub>2</sub>. Included in the reactions concerned are the cleavage (10) and synthesis of serine (11), and the



synthesis from the  $\beta$ -carbon of serine of the methyl groups of choline (12). The isolation of 5-formyl-5, 6, 7, 8-tetrahydrofolic acid from natural sources (13, 14) and its requirement for the growth of *Leuconostoc citrovorum* (15) suggested that this form of the vitamin served as a carrier of these fragments in at least one stage of their metabolism. The "active"  $C_1$  intermediate was assumed to participate catalytically in condensation or cleavage reactions in a manner analogous to that of acetyl coenzyme-A. A formylated coenzyme could be reasonably postulated in the synthesis of purines (carbons 2 and 8), or in the utilization of imidazole-C-2 of histidine (16), indole- $\alpha$ -carbon of tryptophan via N'-formyl-L-kynurenine (17), and administered formate. However, the observation that C—H bonds are not broken in the overall conversion of the hydroxymethyl group of serine to methyl indicates that formate or formyl derivatives of folic acid are not obligatory intermediates in this process.

These results do not exclude other derivatives of folic acid as coenzymes. The postulation of an N-5-hydroxymethyl, or closely related, derivative of tetrahydrofolic acid would be in agreement with the observed stability of the  $\beta$ -hydrogen atoms of serine. This stability is further illustrated by the unaltered  $C^{14}$ :D ratio of internal organ serine following the administration of serine- $\beta$ - $C^{14}D_2OH$  (18). In the experiments reported in Table 1 the  $C^{14}$  and D ratios of the visceral protein serine were the same as those in the labeled serine administered, even though an extensive cleavage and resynthesis of serine must have been going on. Similar results were obtained in the conversion of  $DC^{14}OONa$  to serine. When either labeled serine or formate was given (6) the deuterium of C-2 and C-8 of purines, however, was found to be extensively labilized (Table 3). It would appear then, that, if exchange of D occurred in a derivative of folic acid, two types of folic acid coenzyme may be envisioned. The interrelationships between the "formyl" and "hydroxymethyl" intermediates are formulated provisionally in Fig. 1.

Since D from  $DC^{14}OONa$  and serine- $\beta$ - $C^{14}D_2OH$  is labilized in the synthesis of purines, whereas it is not labilized in the synthesis of serine and methyl groups, the reversal of reaction 4 does not

TABLE 3  
UTILIZATION OF L-SERINE-3-C<sup>14</sup>,D AND  
DC<sup>14</sup>OONa FJR C-2 or C-8 OF ADENINE (9)

Compound fed	Dilution		Ratio of dilutions (C <sup>14</sup> /D *)
	C <sup>14</sup>	D	
Serine I A	29	100	0.29
B	25	53	0.47
Serine III	48	190	0.25
DC <sup>14</sup> OONa	31	90	0.35

\* In a sample of guanine from Serine I a ratio of dilutions of C<sup>14</sup>/D = 37/178 = 0.2 was found for C-8.

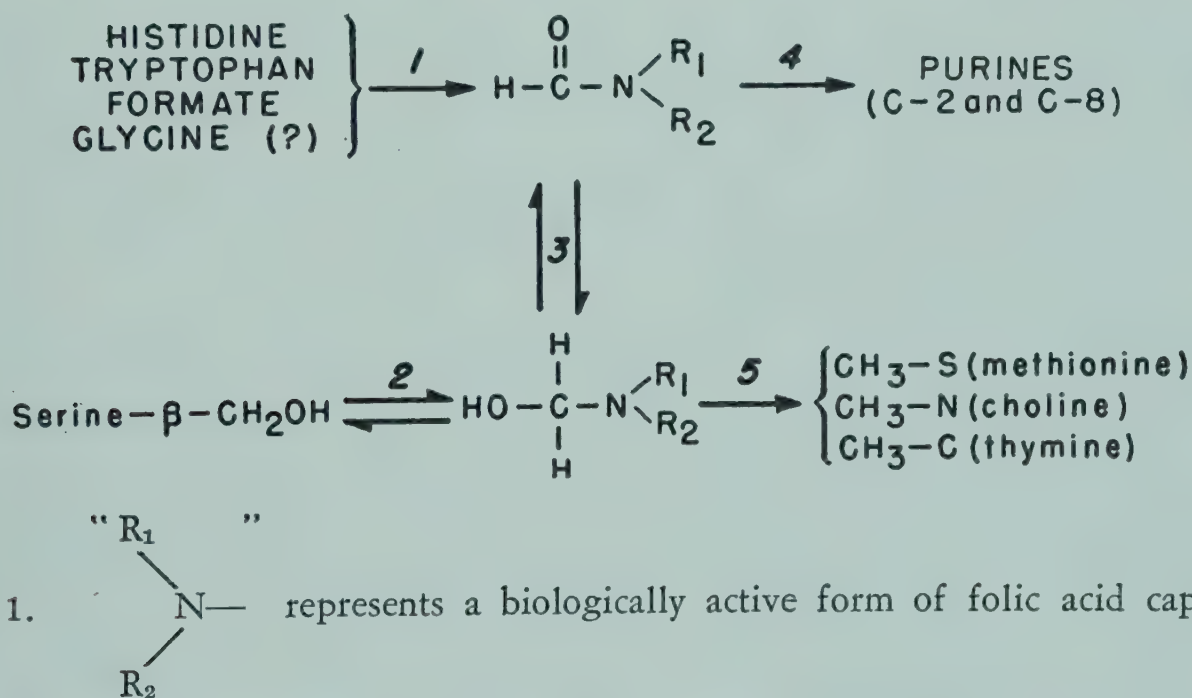


FIG. 1.  $\begin{matrix} \text{“R}_1\text{”} \\ \diagdown \\ \text{N—} \\ \diagup \\ \text{R}_2 \end{matrix}$  represents a biologically active form of folic acid capable of

holding C<sub>1</sub> either on a level of oxidation of “formate” or “formaldehyde.” Arrows are meant to indicate not single reactions but processes which may involve several steps.

appear to be quantitatively important in the intact animal under our experimental conditions. Although reaction 3 should be reversible, the stability of D in the conversion of the β-carbon of serine to methyl groups would not be appreciably affected if serine is the major source (70 per cent) of newly formed methyl groups (19), and reaction 3 is not faster than reactions 4 and 5.



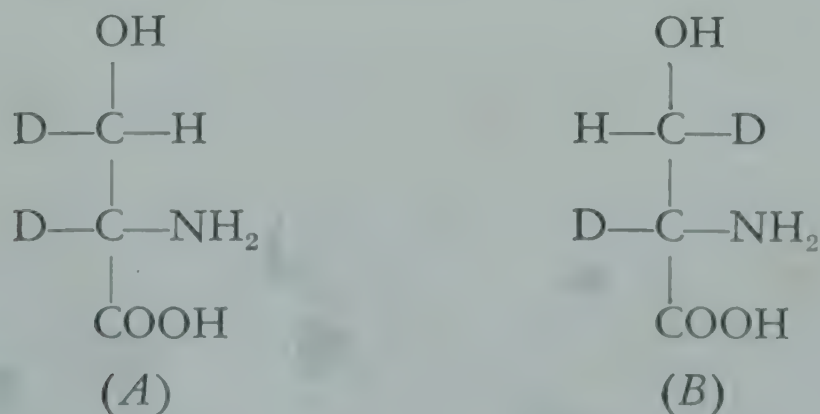
Evidence for a "hydroxymethyl" intermediate in the metabolism of serine has been obtained recently in the conversion of the methyl group of sarcosine to the  $\beta$ -carbon of serine (20), in the synthesis of serine from glycine and in the exchange reaction:  $\text{CH}_2\text{OH} \cdot \text{CHNH}_2 \cdot \text{COOH} + \overset{+}{\text{CH}_2}\text{NH}_2 \cdot \overset{*}{\text{COOH}} \rightleftharpoons \text{CH}_2\text{OH} \cdot \overset{+}{\text{CH}}\text{NH}_2 \cdot \overset{*}{\text{COOH}} + \text{CH}_2\text{NH}_2 \cdot \text{COOH}$ . In the latter two reactions, tetrahydrofolic acid (21, 22, 23) had a marked stimulating effect.

The postulation of an active  $\text{C}_1$  intermediate capable of existence on a level of oxidation of either aldehyde or acyl is analogous to the occurrence of a  $\text{C}_2$  intermediate as active acetaldehyde and active acetyl (24).

#### THE POSSIBLE EFFECTS OF SELECTIVE CLEAVAGE

The conclusions presented here are valid only if selective cleavage of carbon-protium as opposed to carbon-deuterium bonds does not occur. Selection could take place in molecules of serine containing only one atom of D attached to the  $\beta$ -carbon. It could be due to either steric or kinetic effects.

The synthesis of Serine I (see first footnote to Table 1) by addition of  $\text{D}_2$  to ethyl formylhippurate,  $\text{HC}(\text{OH}) = \text{C}(\text{NHCOPh}) \cdot \text{COOEt}$ , was such that none of the molecules contained more than one atom of D in the  $\beta$ -carbon, and only one of the two stereoisomers of L-serine-2,3-D might have been produced, *A* or *B*.



Since the ratio of  $\text{C}^{14}:\text{D}$  in the methyl groups was unaltered, preferential removal of deuterium did not take place. On the other

hand, oxidation of the  $\beta$ -carbon of serine to the level of formate, during methyl group synthesis, might have occurred by preferential removal of protium. This could have been due to the greater strength of C—D than of C—H bonds, or to the stereospecificity of the enzymes involved. Although isomers *A* and *B* would both be subject to enzymatic attack, it might be expected that an atom of D would be removed from *A*, while an atom of H would be removed from *B*, or vice versa. Similar considerations would apply if oxidation occurred after cleavage of the serine molecule.

Serine II was synthesized to test the possibility of steric selection. Deuterium was introduced in such a way that if Serine I consisted of only one of the two stereoisomers, Serine II would consist of only the other. Although the amount of deuterium in Serine II was too low to give a desirable level of isotope in the methyl groups, the results obtained with this material (Table 1) tended to confirm the findings with Serine I, and to suggest that steric factors are not responsible for the unaltered  $C^{14}$ :D ratios.

Serine III was prepared to test both steric and kinetic effects. In this compound 1.41 atoms of D were attached to the  $\beta$ -carbon in a random manner, so that 50 per cent of the molecules contained two atoms of D, 40 per cent one atom each of D and H, and 10 per cent 2 atoms of H. Selective cleavage from whatever cause could apply only to the 40 per cent of the molecules containing one D and one H. Molecules containing two atoms of D would have to lose one if the conversion of serine to methyl groups proceeded by way of "formate" or its derivatives. If in this process preferential breaking of C—H bonds were involved to any considerable extent, the methyl groups derived from Serine III should show a higher  $C^{14}$ :D ratio than was observed with Serine I. Such was not the case. The ratio of  $C^{14}$  dilution to D dilution is fairly close to 1 irrespective of the type of serine used. It is, therefore, unlikely that either steric or kinetic effects are responsible for the observed stability of deuterium in the synthesis of methyl groups from serine.



THE RELATIVE UTILIZATION OF SERINE AND GLYCINE  
FOR C<sub>1</sub> FRAGMENTS

In order to equate the variable dosage and activity of the several precursors investigated, the observed isotope dilutions were converted to coefficients of utilization,<sup>1</sup> and compiled in Table 4.

TABLE 4  
RELATIVE UTILIZATION OF C<sub>1</sub> PRECURSORS

Precursor	Coefficient of utilization (25) for			
	Serine ( $\beta$ -carbon)	PNA Guanine (C-2 or 8)	Thymine (methyl)	Choline (methyl)
Serine-3-C <sup>14</sup>	50	42	21	7
Formate-C <sup>14</sup>	9	35	9	4
Glycine-2-C <sup>14</sup>	11	4	3	1
Histidine-2-C <sup>14</sup> (16)	12	22		3

The  $\beta$ -carbon of serine is incorporated nearly equally into proteins and C-2 + 8 of PNA guanine, whereas "formyl" precursors, such as histidine (16) and formate, are much more effective precursors of purines than of protein serine. Compared to serine, the other compounds (glycine, histidine, and formate) are poor precursors of methyl groups. Glycine, however, differs from histidine and formate since it is also poorly utilized for C-2 + 8 of purines. Although classed with "formyl" precursors in Fig. 1, it would appear that glycine- $\alpha$ -carbon more readily enters into equilibrium with serine or " $R_1(R_2)N-CH_2OH$ " than with formate or " $R_1(R_2)N-CHO$ " (cf. 19). Histidine and formate, however, are more closely related to the " $R_1(R_2)N-CHO$ " pathway.

<sup>1</sup> The coefficient of utilization (25) is derived from the isotope dilution formula  $b = a[(x/y) - 1]$ , where  $a$  = millimoles of labeled compound administered per 100 g. per day,  $b$  = millimoles of material per 100 g. per day elaborated by the organism and used to dilute  $a$ ,  $x$  = isotope concentration of  $a$ , and  $y$  = isotope concentration of the compound isolated. The coefficient of utilization =  $1000/b = 1000/a[(x/y) - 1]$ . It is expressed as 1000 times the reciprocal of  $b$  so that it will show an increase with increasing utilization. As modified for use with isolated tissue constituents,  $a$  becomes the total rather than the daily dose.

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# METHIONINE DEGRADATION BY A SPECIES OF *PSEUDOMONAS*

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BY MEANS OF the enrichment technique a number of strains of the genus *Pseudomonas*<sup>1</sup> were isolated which were capable of growing in a medium containing DL-methionine as the sole carbon and nitrogen source. The utilization of methionine by one strain was investigated in some detail in regard to the primary reactions involved in the degradation of the amino acid molecule.

Thoroughly washed suspensions of cells grown at the expense of DL-methionine oxidized the amino acid with the uptake of 2.7  $\mu$ moles of oxygen per mole of methionine, ammonia and dimethyl disulfide appearing as end products. No other end-product of the oxidation having been detected, far-reaching oxidation or assimilation of the molecule is indicated. The addition of arsenite to resting cells oxidizing methionine results in considerable inhibition of the oxygen consumption, and under these conditions only 0.5 micromoles of oxygen are consumed per micromole of substrate; one  $\mu$ M. of ammonia accumulates per  $\mu$ M. substrate, and a keto acid appears in the medium. Fig. 1 illustrates such oxygen consumption data. The keto acid was identified as  $\alpha$ -keto- $\gamma$ -methiobutyric acid by its chromatographic behavior when a solvent phase of water-saturated butanol, 95 parts: propionic acid, 5 parts, was used (1). Following the migration the keto acid was developed by spraying with a 1% aqueous solution of *p*-hydrazinobenzenesulfonic acid and was read under ultraviolet light. Under these conditions  $\alpha$ -keto- $\gamma$ -methiobutyric acid ( $\alpha$ -ketomethionine) has an  $R_f$  of 0.41. Further identification was afforded by the isolation of the keto acid as the 2,4-dini-

<sup>1</sup> A full account of the characteristics of these organisms will appear elsewhere.

trophenylhydrazine and by determination of the sulfur content of the derivative ( $S = 9.68\%$ ; theoretical  $S = 9.76\%$ ). There was no depression of mixed melting point with an authentic derivative. (m. p. =  $147-148^\circ$ , uncorrected).

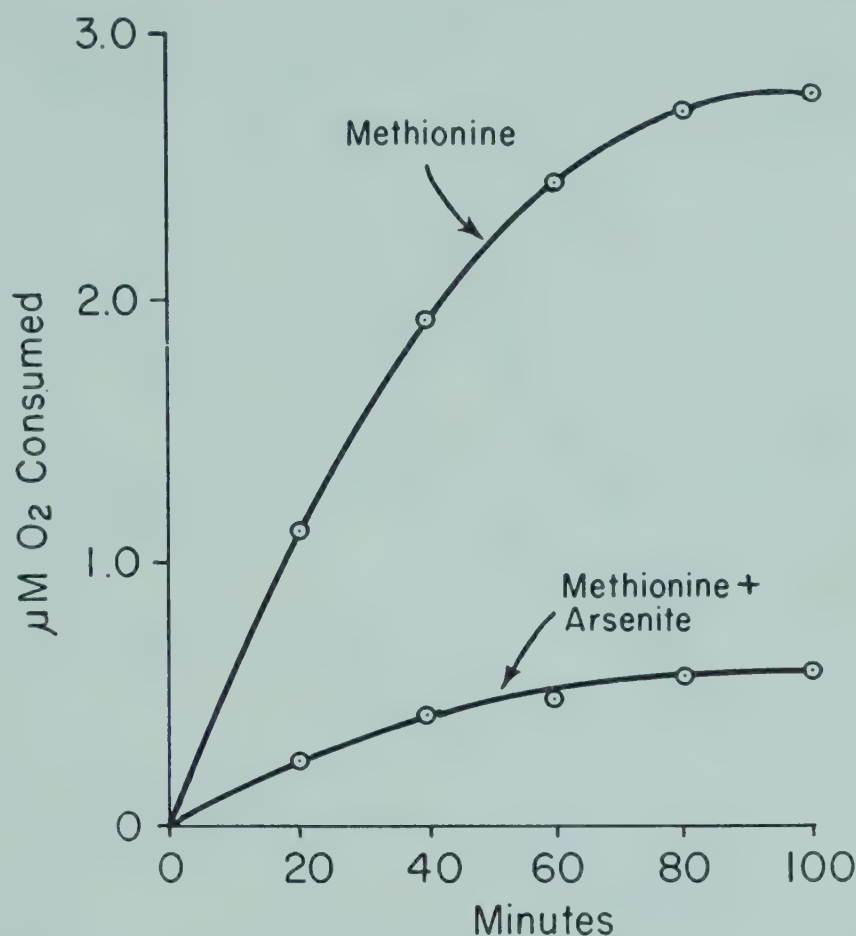


FIG. 1. Oxidation of L-methionine by washed suspensions of DL-methionine-grown cells. Temp =  $30^\circ$  C.; pH = 7.2; 4 mg. dry weight cells;  $1.0 \mu M$ . substrate. Endogenous oxygen uptake subtracted.

In the case of cells grown at the expense of the L isomer, D- and L-methionine oxidation data assumed the patterns shown in Fig. 2. A somewhat higher level of substrate ( $10 \mu M$ .) was used, and under such conditions the curve of L-methionine oxidation is seen to rise steeply, break, and thereafter continue its rise at a somewhat slower rate. The break invariably occurs when  $0.5 \mu M$ . oxygen per  $\mu M$ . L-methionine has been consumed. Not plotted on the curve is the fact that ketomethionine accumulates during the early phases of the oxidation ( $7.7 \mu M$ . at 30 minutes) and gradually disappears as the process continues ( $6.6 \mu M$ . at 1 hr.;  $3.1 \mu M$ . at 2 hr.). No other keto acid is excreted during the process. These data led us to suspect that the C—S bond of methionine underwent cleavage only after



the amino acid was oxidatively deaminated to the keto analogue, but this concept was not borne out by later experimentation. In the presence of 0.001 *M.* arsenite, one mole of ketomethionine accumulates per mole of methionine utilized.

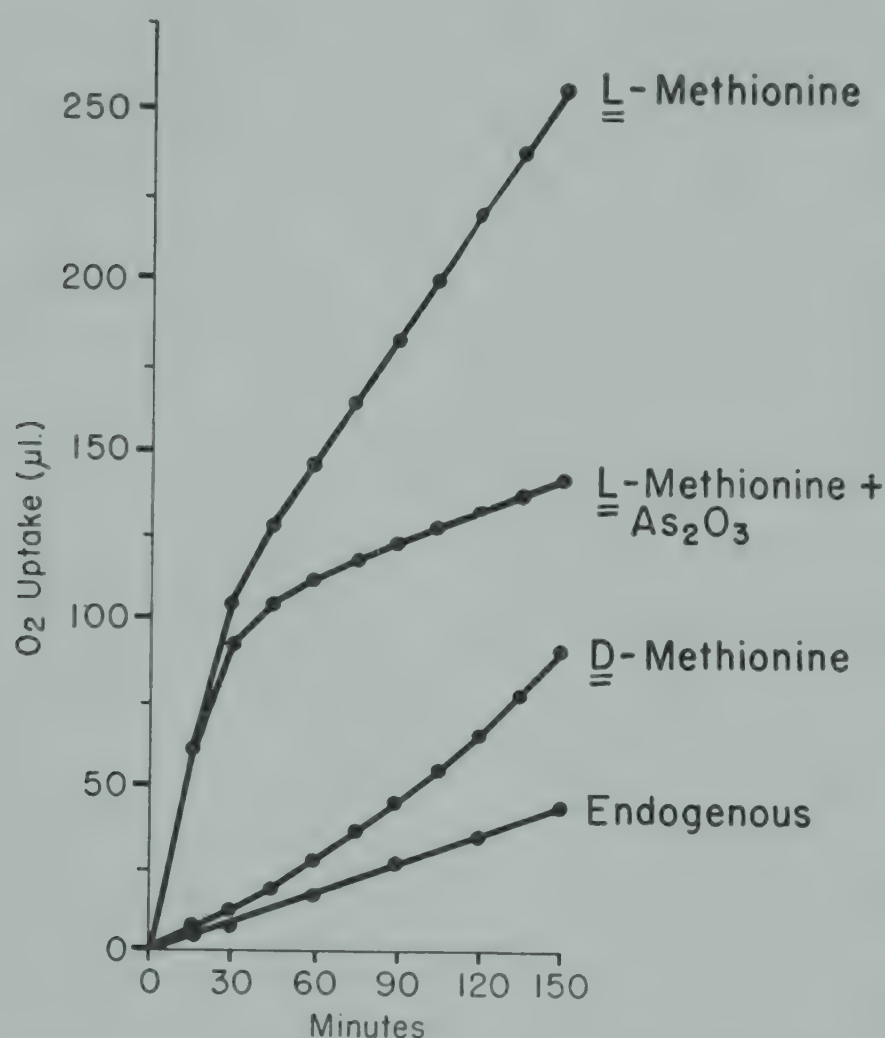


FIG. 2. Oxidation of L- and D-methionine by suspensions of L-methionine-grown cells. Conditions as in Fig. 1, except 10  $\mu$ M. substrate present.

The fact that only dimethyl disulfide can be isolated from growing cultures and from resting cells oxidizing methionine deserves some comment. The Grote modification of the nitroprusside test (2) differentiates between —SH and —S—S— groups, and application of the test indicated that at no time was methyl mercaptan produced by these cells; rather, the disulfide (oxidation product) was invariably detected. Methyl disulfide was isolated and identified as the mercuric chloride complex which forms when  $\text{CH}_3\text{—S—CH}_3$  in excess reacts with mercuric chloride. Reductive decomposition of this complex with Zn-HCl mixtures yielded a volatile compound which formed, when bubbled through mercuric cyanide solution,

a mercuric derivative that after recrystallization from alcohol yielded a white solid melting at 181-183° C. (uncorr.). There was no depression of mixed melting point upon admixture with an authentic mercuric salt derivative (m. p. = 182-184° C.). Methyl mercaptan produced from reduction of the mercuric chloride complex was also identified as the 2, 4-dinitrophenylthioether derivative (m. p. = 126-128° C., uncorr.). Furthermore, the gases evolved from aerated growing cultures or aerated resting cell suspensions oxidizing methionine, when passed into a weakly alkaline nitroprusside solution, failed to elicit the characteristic color produced by RSH unless first bubbled through NaCN solution, thereby cleaving the —S—S— bond. It was concluded that no methyl mercaptan is produced aerobically by this organism from methionine. It may, with considerable justification, be argued that under the experimental conditions employed the mercaptan is rapidly autooxidized to the disulfide. Against this argument must be balanced the observations made by Challenger and Charleton (3) and by Segal and Starkey (4), who found that a variety of fungi and soil bacteria produced both methyl mercaptan and dimethyl disulfide from methionine under somewhat similar experimental circumstances. In any event, the oxidation of methyl mercaptan would account for only 0.25  $\mu$ M. of oxygen per  $\mu$ M. of methionine.

### ENZYMATIC STUDIES

Extracts of organisms grown in a yeast-extract-methionine medium (aerobically for 28 hours) were prepared by sonic oscillation. Following the removal of cell debris by centrifugation, the supernatant extract could be fractionated by standard methods into three fractions provisionally called the L-amino acid oxidase fraction, methionine dethiomethylase, and methionine racemase. Table 1 outlines the essential steps in the fractionating procedures.

*L-amino acid oxidase fraction.* Precipitation of the crude cell-free extract with 30 per cent saturated ammonium sulfate yields a precipitate which, on redissolving in 0.05 M. phosphate buffer (pH 7.4)



TABLE 1

FRACTIONATION OF PSEUDOMONAS EXTRACT FOR L-AMINO ACID OXIDASE,  
METHIONINE RACEMASE, AND METHIONINE DETHIOMETHYLASE

Suspend 7.0 grams, wet weight, of cells in 30 ml. 0.1 M. phosphate buffer, pH 8.2; subject to sonic oscillation for 25 minutes in the cold; centrifuge	
Resuspend in 10 ml. 0.1 M. phosphate buffer, pH 8.2; subject to sonic oscillation 25 minutes; centrifuge	
Discard cell debris	Combine supernatants; add solid $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation; centrifuge
Dissolve in 0.1 M. phosphate buffer, fractionate by differential centrifugation (Stumpf and Green, 1944). This fraction contains bulk of <i>L-amino acid oxidases</i> .	Add solid $(\text{NH}_4)_2\text{SO}_4$ to 75% saturation; centrifuge
Dissolve in water; adjust to pH 6.0, add protamine sulfate to $1280 \text{ m}\mu/1260 \text{ m}\mu = 0.97$ ; centrifuge	Discard
Discard	Add solid $(\text{NH}_4)_2\text{SO}_4$ to 35% saturation; centrifuge
Discard	Add solid $(\text{NH}_4)_2\text{SO}_4$ to 55% saturation; centrifuge
Dissolve in phosphate buffer, pH 7.4. <i>Dethiomethylase</i>	Add solid $(\text{NH}_4)_2\text{SO}_4$ to 75% saturation; centrifuge
Dissolve in 0.01 M. phosphate buffer, pH 8.2, adsorb on $(\text{Ca})_3(\text{PO}_4)_2$ gel (100 mg. gel/100 mg. protein). Centrifuge	Discard
Wash with two portions 0.01 M. phosphate buffer, pH 8.2; centrifuge	Discard
Elute with two portions 1.0 M. phosphate buffer, pH 8.2, for three hours in the cold; centrifuge	Discard
Discard	Dialyze overnight against distilled water at $0^\circ \text{C}$ . <i>Methionine racemase</i>

will oxidize L-methionine. Quantitatively 0.5  $\mu$ M.  $O_2$  are consumed per  $\mu$ M. methionine utilized. Some other quantitative aspects of the L-amino acid oxidase system are recorded in Table 2. The keto acid which is shown to accumulate was isolated as the 2,4-dinitrophenylhydrazone and identified as ketomethionine both by melting point and chromatographic behavior. The melting and mixed melting points carried out with an authentic derivative were 147-149° C. (uncorr.), corresponding quite well with values reported in the literature (5).

TABLE 2

**L - Amino Acid Oxidase in  
*Pseudomonas spp.***

Substrate	$O_2:NH_3$ : Keto Acid / Mole Substrate Consumed
L- Methionine	0.5 : 1.1 : 0.9
D- Methionine	0.0 : 0.2 : 0.0
L- Methionine + 0.001 M. Arsenite	0.5 : 1.0 : 0.9

SUBSTRATE = 2  $\mu$ M  
pH = 7.4 (PHOSPHATE)  
TEMP. = 30°C  
ENZYME = 0.2 mg. PROTEIN

Like the L-amino acid oxidase described by Stumpf and Green (6), the enzyme showed marked stereochemical specificity, only the L isomer being oxidized. In view of the crude nature of the preparation it is not surprising that a variety of L-amino acids are oxidatively deaminated. No hydrogen peroxide formation (7) was ever detected in the reaction mixtures. High speed centrifugation ( $50,000 \times g$  for 90 minutes) considerably reduced the endogenous oxidation with a concomitant increase in specific activity, but no change was detected in the amino acid specificity or stereospecificity of the preparations. The obvious conclusion is that this fraction contains a variety of L-amino acid oxidases similar to, if not identical with, those described by Stumpf and Green. It may be pointed out that arsenite at a level of 0.001 M. has no inhibiting effect on the amino acid oxidase.



*Methionine dethiomethylase.* Following the removal of nucleic acid by the protamine sulfate method of Korkes et al. (8), a fraction precipitating between 35 per cent and 55 per cent saturation with ammonium sulfate was found to attack L-methionine anaerobically. The reaction products of the enzymatic attack on methionine were identified as  $\text{NH}_3$ ,  $\text{CH}_3\text{SH}$ , and  $\alpha$ -ketobutyric acid. Identification of the keto-acid was accomplished by chromatography (vide supra) and by isolation of the 2,4-dinitrophenylhydrazone derivative, which

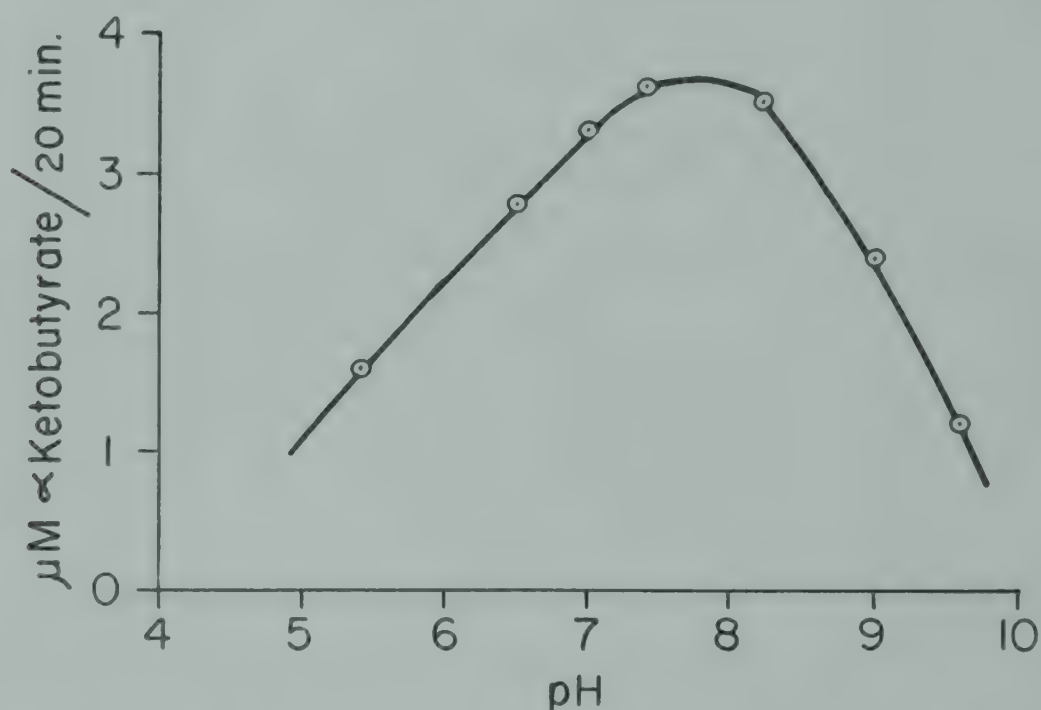


FIG. 3. Effect of pH on methionine dethiomethylase. 20  $\mu\text{M}$ . substrate, 4  $\gamma$  pyridoxal phosphate, 0.1 M. phosphate buffer, 0.2 ml. enzyme. Total volume = 2.0 ml., incubation at 37° C., anaerobically.

melted at 200-202° C. (uncorr.), the mixed melting point being 200-203° C. (uncorr.). Keto-acid production measured by the Friedmann-Haugan method (9) afforded a convenient assay of enzymatic activity under a variety of conditions, and some properties of the enzyme, provisionally called methionine dethiomethylase (because of its characteristic action in releasing the thiomethyl group) were determined. Experiments were carried out in small test tubes (10  $\times$  70 mm.) containing substrate, buffer, and enzyme, generally in a volume not exceeding 2.0 ml. Following the incubation the reactions were stopped with trichloroacetic acid and keto-acid analyses were performed on aliquots of the supernatant. Enzymatic activity is maximal at pH values between 7.4 and 8.0 (Fig. 3). Prolonged

dialysis for 24 to 30 hours in the cold resulted in a very considerable loss of activity, probably by oxidation of the cofactor, but all the activity could be restored by the addition of pyridoxal phosphate (Table 3). The cofactor could also be quantitatively removed by simple adsorption on levigated alumina at a pH of 7.4 in 0.05 *M*. phosphate buffer. Dethiomethylase appears to be identical with the

TABLE 3

**Dethiomethylase Activation by  
Pyridoxal Phosphate**

Preparation	$\mu$ M. Keto - butyrate Produced per Hour
Undialyzed	10.4
Undialyzed + 48 B <sub>6</sub> al-PO <sub>4</sub>	11.7
Dialyzed	2.0
Dialyzed + 48 B <sub>6</sub> al-PO <sub>4</sub>	11.0

L-METHIONINE = 40  $\mu$ M  
pH = 7.4

ENZYME = 0.2 mg PROTEIN

methionine demercapto-deaminase in *Clostridium sporogenes*, recently described by Wisendanger and Nisman (10), but its exact relationship to other systems capable of breaking the —C—S— bond has not yet been clearly established.

Methionine dethiomethylase appears to have a high order of stereochemical specificity; only the L enantiomorph undergoes degradation as judged by the —SH test or keto-acid production. The stereochemical specificity is the same whether the cells have been grown on L- or DL-methionine, although intact cells grown on DL-methionine will attack both isomers. Despite the fact that methionine was attacked by this system there still remained the possibility that rupture of the —C—S— bond occurred only after a degradation with acceptors other than oxygen to yield ketomethionine, following which methyl mercaptan was released by another mechanism. In order to investigate this possibility  $\alpha$ -keto methionine was prepared by the method of Meister (11) using D-methionine and purified



D-amino acid oxidase from hog kidney (12). Since little or no ketobutyrate was formed from ketomethionine and since the  $\text{HSCH}_3$  tests were negative, it was concluded that dethiomethylase actually attacks the intact methionine molecule. In this regard dethiomethylase differs from a bacterial enzyme, described by Mitsuhashi (13), which produces  $\text{HSCH}_3$  from both methionine and ketomethionine, although the latter yields only 30 per cent as much  $\text{HSCH}_3$  under similar conditions as does methionine.

Arsenite at a final concentration of 0.001 *M*. completely inhibits the activity of methionine dethiomethylase. A like situation with respect to arsenite is found in the desulfhydrase systems of *Proteus morganii* which produce pyruvate and  $\alpha$ -ketobutyrate, respectively from cysteine and homocysteine, with a concomitant production of  $\text{NH}_3$  and  $\text{H}_2\text{S}$  in both cases (14). Methionine does not appear to undergo degradation by the *P. morganii* desulfhydrases. Cysteine desulfhydrase from rat liver is also inhibited by arsenite (15). A close relationship between homocysteine desulfhydrase and methionine dethiomethylase is clearly possible, since both are activated by pyridoxal phosphate (16), but whether or not they are identical will have to await information from further comparisons.

*Methionine racemase.* In the enzyme fraction precipitating between 55 and 65 per cent saturation with ammonium sulfate is added to dethiomethylase, a preparation results which will produce methyl mercaptan and  $\alpha$ -ketobutyrate from both optical isomers of methionine. A number of possibilities presented themselves, among which the following were considered:

- (1) If the split of the C—S bond occurs in ketomethionine, a D-amino acid dehydrogenase might be functioning to provide ketomethionine for dethiomethylase action.
- (2) A methionine racemase may be present in the second fraction.
- (3) D-amino acid transaminase might operate on endogenous amino acceptors (e. g. pyruvate) in conjunction with alanine racemase and L-amino acid transaminase, the net result of which would be production of the L isomer from the D form of the substrate.



The finding previously noted, that dethiomethylase does not attack ketomethionine, eliminates the first possibility. In the event that a racemase were operating it should be possible to demonstrate the formation of one optical isomer from a pure substrate of the other. Such an experiment was devised, using D-methionine incubated anaerobically with the enzyme preparation, and periodically assays were performed manometrically for L-methionine, using L-amino acid oxidase prepared from lysates of *Proteus vulgaris* as outlined by Stumpf and Green (6). Racemization of D-methionine was measured in small test tubes containing 15  $\mu$ M. D-methionine, phosphate buffer, and 0.5 ml. of a crude racemase preparation in a total volume of 2.0 ml. After anaerobic incubation for the requisite time, the reaction was stopped by brief immersion of the tubes in boiling water, the protein was centrifuged off, and a 1.0 ml. aliquot was removed for a test of the L-methionine content. Measurements for L-amino acid were performed by conventional Warburg manometry using 0.5 ml. of the amino acid oxidase preparation. Results from one such experiment are shown in Fig. 4. It is quite evident that L-methionine accumulates to almost the theoretical level of a racemic mixture. In view of the fact that the L-amino acid oxidase preparations unquestionably were mixtures of many enzymes, subsequent experiments were carried out by assaying for the formation of the D isomer from L-methionine with purified hog kidney D-amino acid oxidase. By following the enzyme some further separation from other protein was effected by adsorption on and elution from a calcium phosphate gel. Maximal activity of the latter preparations required the addition of pyridoxal phosphate, a requirement similar to that of alanine racemase (17). Both L- and D-methionine are racemized by the purified methionine racemase preparations. Such data are shown in Fig. 5, both with and without cofactor additions to the enzyme. The data in Fig. 4 represent the levels of D-methionine which obtain on anaerobic incubation of purified racemase preparations (0.2 mg. protein), with pyridoxal phosphate as indicated, either L- or D-methionine (15  $\mu$ M.) and 0.1 M. phosphate buffer in a total volume of 2.0 ml., as already described. The assay



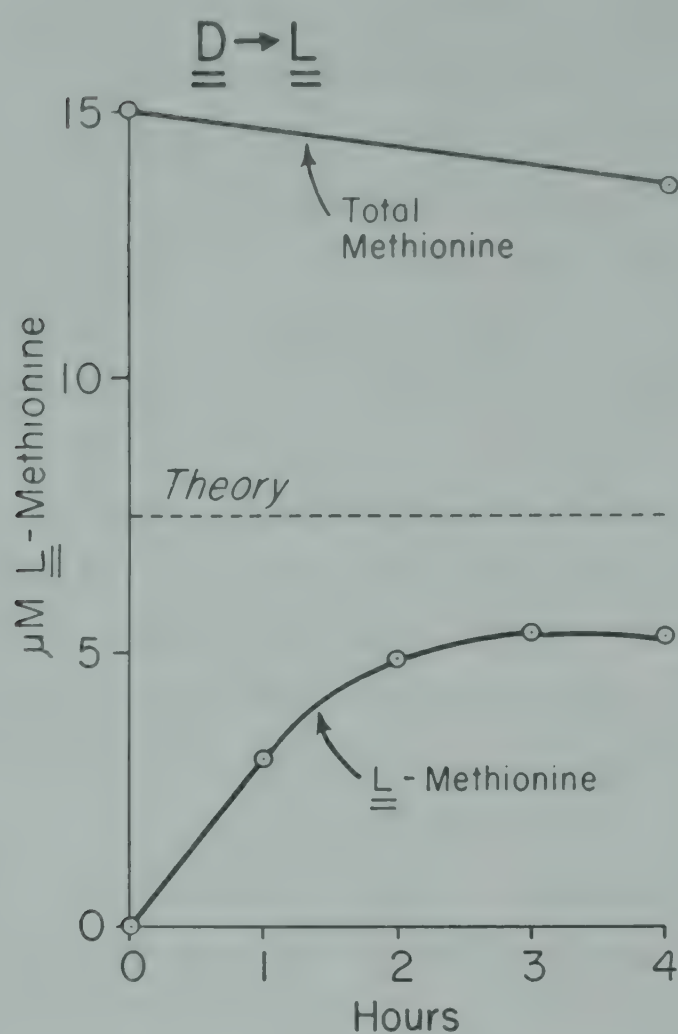


FIG. 4. Methionine racemase activity demonstrated with L-amino acid oxidase from *Proteus vulgaris*. Conditions stated in the text.

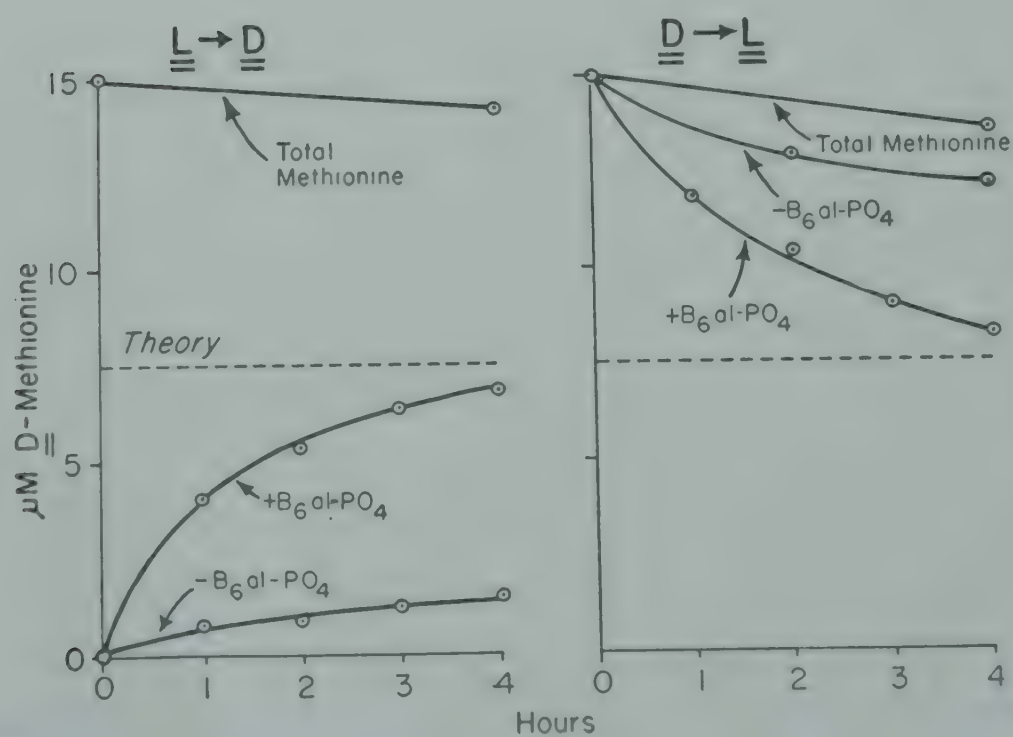


FIG. 5. Methionine racemase activity demonstrated with purified hog kidney D-amino acid oxidase. Conditions stated in the text.

for D-methionine was carried out as detailed by Wood and Gunsalus (17). A number of other properties of the racemase system have been investigated. For example, the effect of pH on the system is shown in Fig. 6, from which it is evident that the optimal pH value

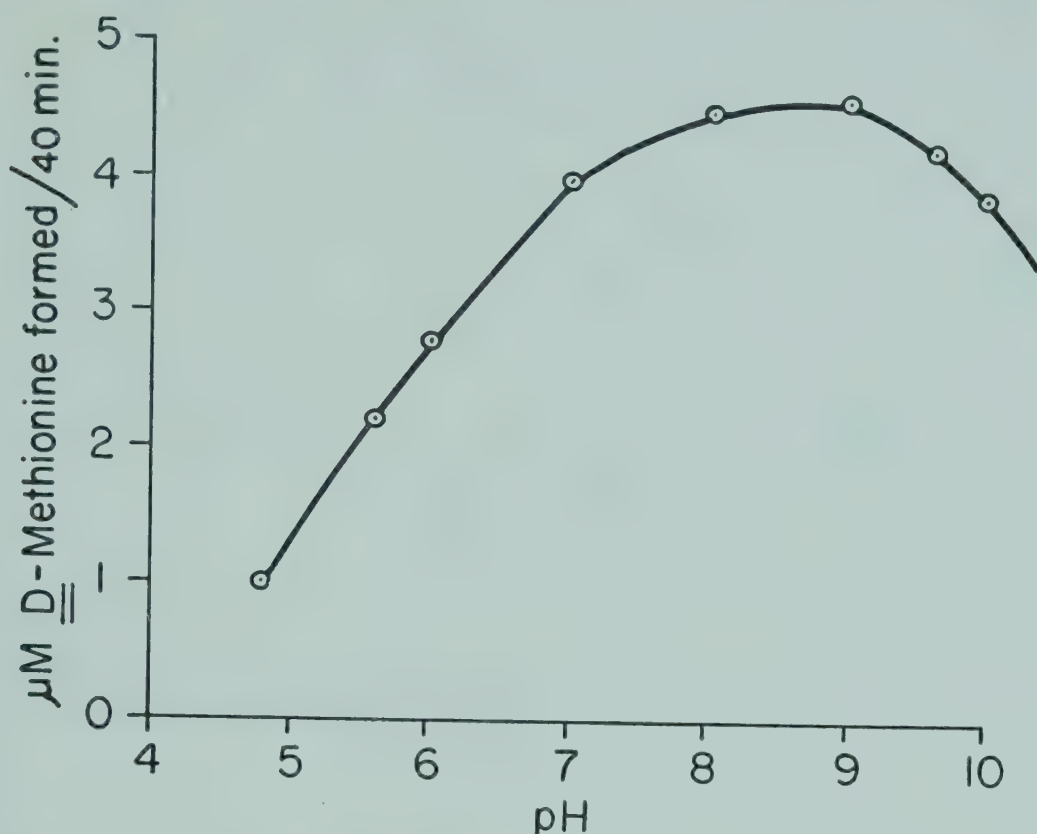


FIG. 6. Effect of pH on methionine racemase. 0.2 mg. racemase preparation, 20  $\mu$ M. L-methionine, 10  $\gamma$  pyridoxal phosphate, phosphate buffer (0.1 M.) in a total volume of 2.0 ml. At the termination of 40 minutes of anaerobic incubation D-amino acid assays were carried out on deproteinized aliquots.

lies between 8.0 and 9.0 (cf. 17). Substrate and cofactor saturation curves are shown in Figs. 7 and 8, respectively. In all aspects studied thus far the enzyme resembles the alanine racemase of Wood and Gunsalus. The half saturation level (Michaelis-Menten's  $K_s$ ) derived from linear plots of experimental data by the method of Lineweaver and Burk is of the order of  $8.0 \times 10^{-3}$  M., a figure in remarkable agreement with the corresponding constant for the alanine-racemizing system.

Prolonged dialysis removed virtually all the racemase cofactor, probably by oxidation; the addition of 8  $\gamma$  of pyridoxal phosphate, as indicated by the cofactor saturation curve (Fig. 7), restored full activity. Half maximal velocity is restored by the addition of 1.0  $\gamma$  of the cofactor.



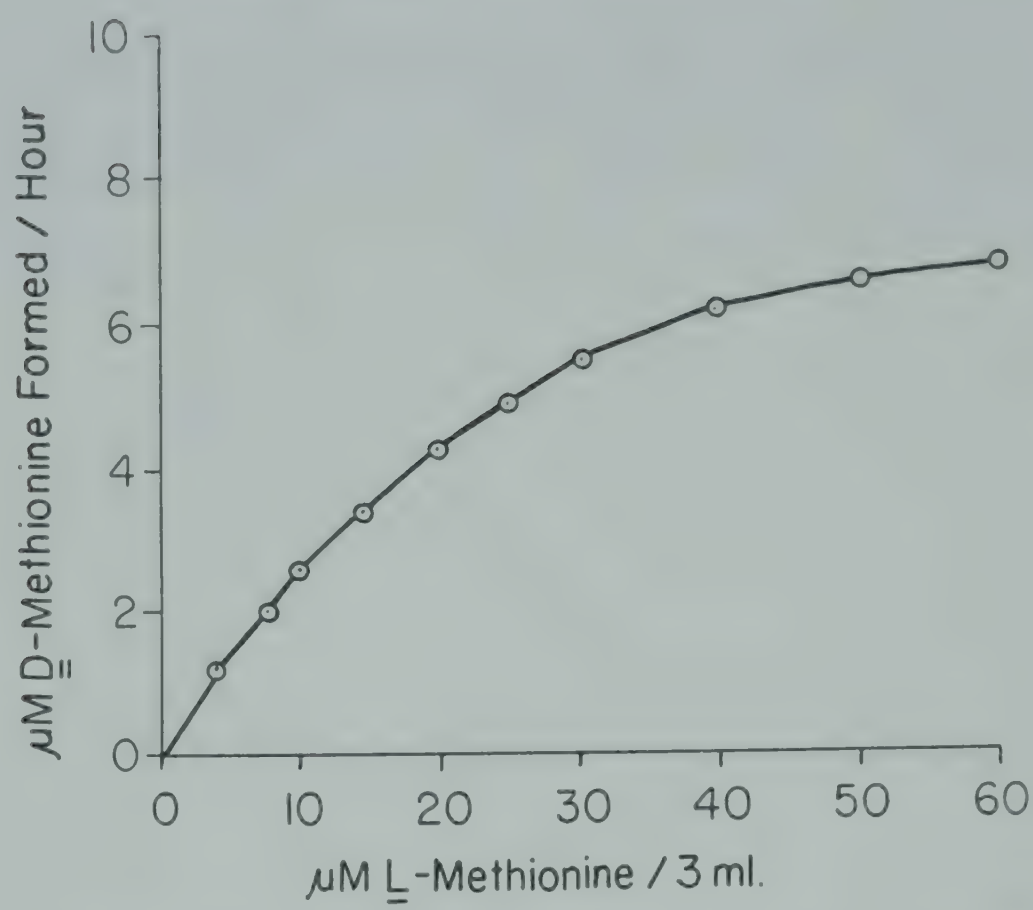


FIG. 7. Substrate saturation curve for methionine racemase substrate as indicated, racemase (0.2 mg. protein), 10  $\gamma$  pyridoxal phosphate, pH 8.2, anaerobic incubation at 30° C.

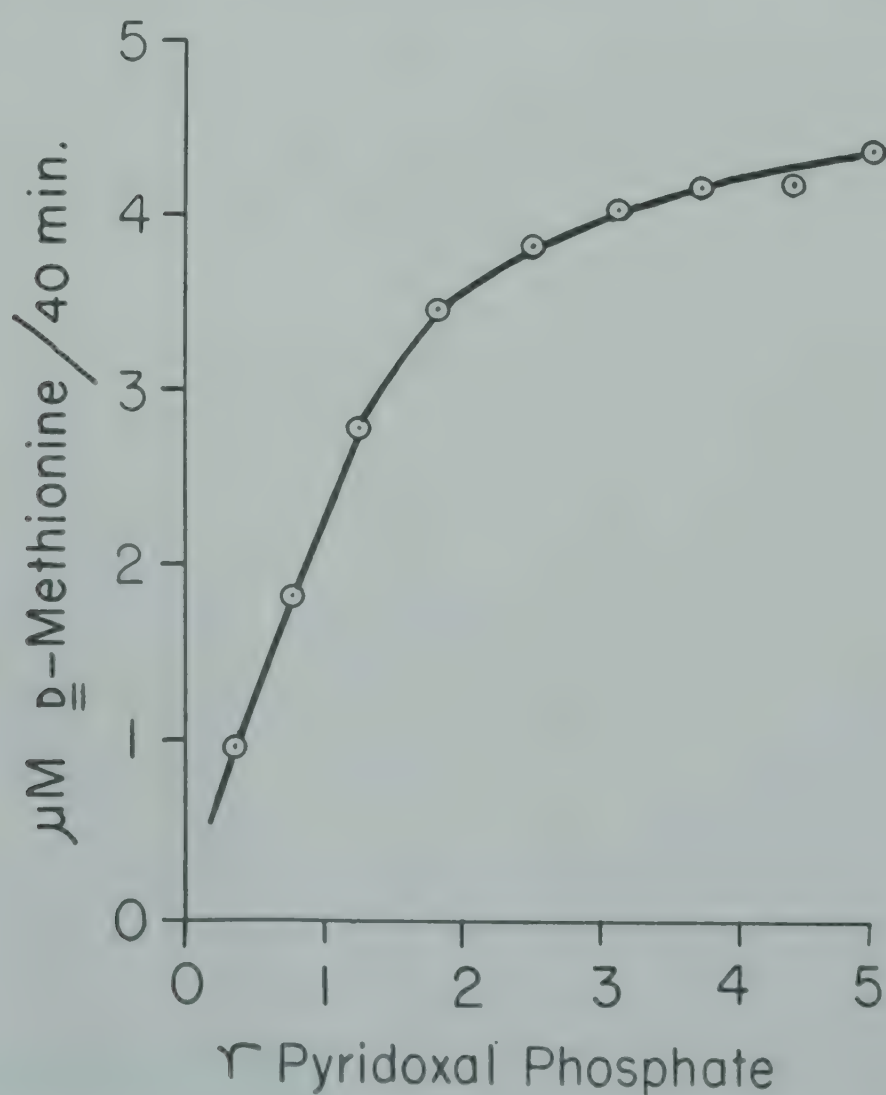


FIG. 8. Cofactor saturation curve for methionine racemase. 20  $\mu\text{M}$ . L-methionine, extensively dialyzed racemase (0.2 mg. protein), 0.1 M. phosphate buffer, pH 8.2, anaerobic incubation for 40 min. followed by D-methionine assay as described in text.

Probable mechanism of the preliminary steps of methionine degradation by the organism. A logical arrangement of the enzymes is that pictured in Fig. 9. In this scheme the oxidative deamination of methionine is presented as a reversible reaction leading essentially

### Metabolism of Methionine By *Pseudomonas* spp.

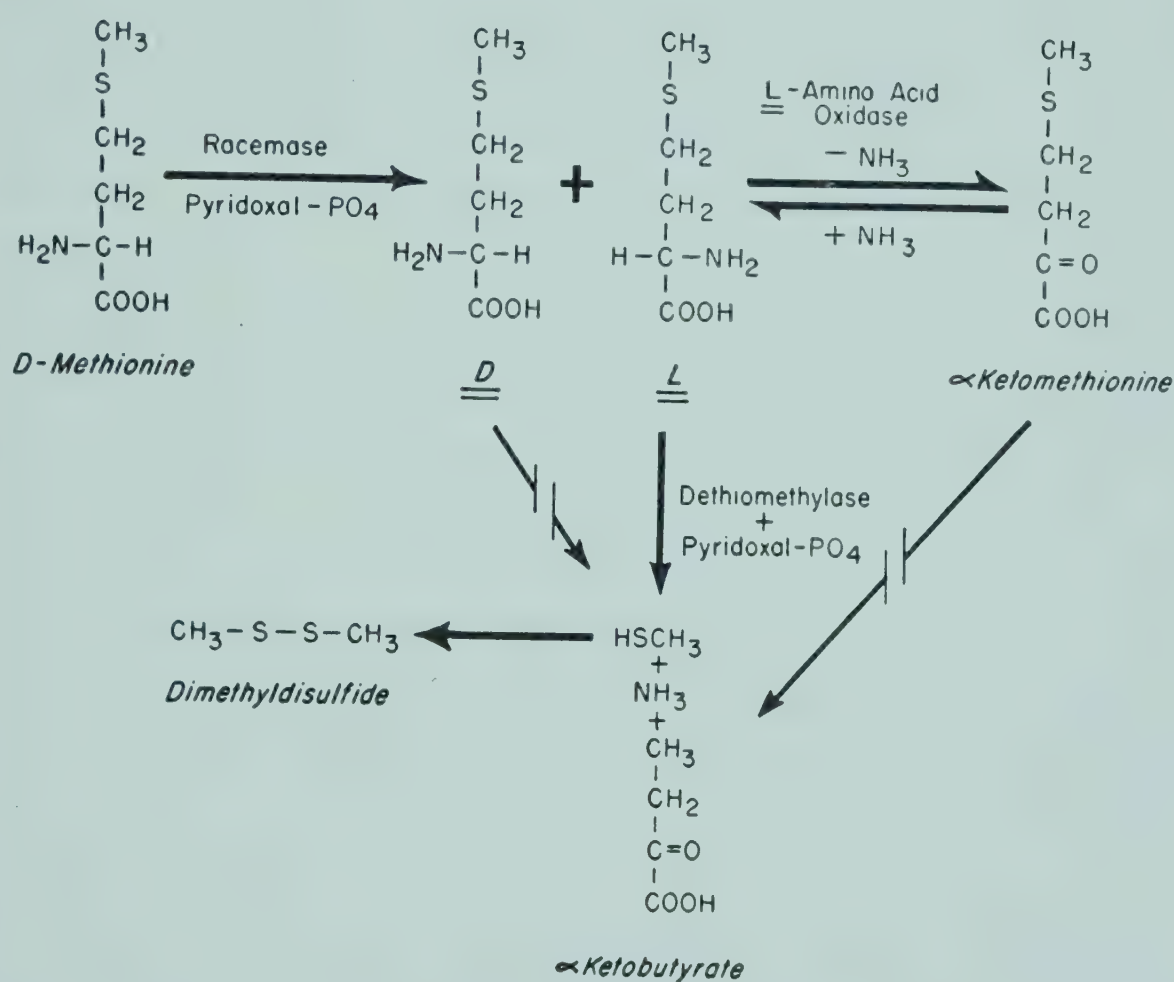


FIG. 9. Pathway of methionine degradation in *Pseudomonas* spp.  
The broken arrows represent reactions which do not occur.

to a dead end. The finding that a variety of heterotrophic organisms isolated by amino acid enrichments (other than methionine) are capable of oxidatively deaminating methionine but are incapable of further attack on ketomethionine lends some support to this thesis. Oxidative deamination is not impaired by arsenite in any of these systems.

The observation that a racemizing system is present in this species of *Pseudomonas* only when growth occurs at the expense of D- or



DL-methionine is somewhat at variance with the finding of Gunsalus, Stanier, and Gunsalus (18). These workers studied mandelic acid racemase in *Pseudomonas fluorescens* and noted that racemase formation in the cells was substrate-induced, but in that case induction of racemase could be accomplished with either optical isomer of mandelate, although it only functioned metabolically when D-mandelic acid was a substrate. There exists another possibility of what is apparently a racemase activity. The recent finding of Thorne (19) that *Bacillus subtilis* extracts contain transaminases specific for D-amino acids suggests the following series of steps which would simulate a methionine racemase action: (1) the transamination between D-methionine and pyruvate to yield D-alanine; (2) racemization of D-alanine by alanine racemase; and (3) transamination of ketomethionine and L-alanine to yield L-methionine. Our finding that nearly the theoretical values were obtained in the racemizing fractions could be interpreted as reflections of equilibria between the various transaminases and alanine racemase as just outlined. A decision between these two possibilities will have to await the results of a more detailed and rigorous examination, now in progress, of the enzymes involved in the racemization of methionine. It may be pointed out, however, that addition of pyruvate or ketomethionine has no effect on the reaction.

Finally, L-methionine is removed by the action of methionine dethiomethylase, a system which appears to be irreversible. More precise methods (cf. Smythe and Halliday, 20) may, however, provide final proof for this question. The oxidation of methionine by this organism is, then, in effect the oxidation of  $\alpha$ -ketobutyrate at which metabolic point both D- and L-methionine eventuate.

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## DISCUSSION

DR. DU VIGNEAUD: In the discussion of labile methyl groups and methyl donors, I should like to remind the group that the term "labile methyl compound" at the time of definition by us was based on the ability of the compound to furnish in the diet methyl groups for various synthetic reactions in the body. Later as a working hypothesis the direct transfer of a methyl group from choline to a methyl acceptor was used by ourselves and many others. It is undoubtedly better to keep this definition from a nutritional viewpoint and not to base it on mechanism, for even if choline must go to betaine to give up its methyl groups, it is still entirely possible that betaine must yet go through some other type of intermediate before its methyl groups are liberated. In other words, it is much more convenient to keep the original definition based on a nutritional concept rather than on a mechanistic basis, for in the latter case one might well be in the position of having to constantly reclassify as one learns more and more about the exact mechanism.

DR. HOROWITZ: I wanted to bring out one or two unpublished results, particularly as they involve a correction of a statement which appears in the literature. Mutants which are blocked in the ability to synthesize methionine are among the commonest kinds of mutants in *Neurospora*. We had at one time approximately 100 independent occurrences of blocks in the synthesis of methionine. As we showed a long time ago, the pathway of methionine synthesis in *Neurospora* from sulfate goes through cysteine to methionine; and, of the 100 mutants, approximately one-half are blocked at some stage between cysteine and methionine, the known intermediates here being cystathionine and homocysteine. The other 50 have blocks in the synthesis of cysteine. A study of the latter group of mutants—that is, between sulfate



and cysteine—has shown that some of them could be satisfied when sulfite was supplied as the sulfur source—that is, they were unable to reduce sulfate to sulfite, whereas others were unable to use sulfate or sulfite but were able to grow when thiosulfate was supplied in the medium. It was also found that cysteic acid could be utilized by just those mutants which are also capable of utilizing sulfite. In other words, cysteic acid and sulfite appear to be equivalent metabolically in this sense, all strains which can use one can use the other, and all the strains which can't use sulfite can't use cysteic acid. It appeared from this that there might be an inorganic pathway of sulfur reduction and an organic pathway. Naturally, one of the compounds which we desired to test was the sulfinic acid. This might be expected to replace thiosulfate in the nutrition of thiosulfate mutants. Phinney reported an experiment a number of years ago at the Genetics Society meeting in which he stated that he had tested preparations of cysteine sulfinic acid and found that it supported the growth of mutants blocked between sulfite and thiosulfate; and he suggested, therefore, that the reduction could occur either by an organic or an inorganic pathway. We have since repeated this experiment with a sample of sulfinic acid which was kindly supplied by Dr. Levene—it is a tricky compound to make—and we found that it does not replace thiosulfate in these mutants. Its availability is identical with sulfite and cysteic acid. It is as if the sulfinic acid is first oxidized to cysteic acid before being utilized, and we have no organic equivalent of the thiosulfate at this time. It has also been found by a former student in our laboratory, Dr. Glenn Fisher, that all of the mutants which are blocked between thiosulfate and cysteine, and which we assumed consisted of a heterogeneous group, some of which were unable to make the final reduction of the sulfur atom to the cysteine level—others which were possibly blocked in the synthesis of the carbon skeleton of cysteine—all of these it turns out are unable to reduce the sulfur atom, because they will all grow when supplied with elemental sulfur. This was a rather surprising observation. Elemental sulfur when mixed with *Neurospora* spores is rapidly converted into sulfide, which is presumably the intermediate. These observations strengthen the notion that the main pathway of sulfur reduction is an inorganic pathway and that the sulfur is reduced before it is attached to the organic skeleton of cysteine. Much, of course, yet remains to be done.

DR. DUBNOFF: No mention has been made here of the possible role of  $B_{12}$  in methionine synthesis. I have suggested that  $B_{12}$  was required for the maintenance of sulfhydryl groups. This was based in part on the fact that homocysteine replaced methionine in the growth of mutant 1133. I would like to present new data on this  $B_{12}$  methionine-requiring mutant, and I would like to show some of the difficulties involved. The mutant seems to appear in several variants, and the growth requirements vary accordingly.



The original mutant required 0.12 millimicrograms of vitamin B<sub>12</sub> for half maximal growth, but it does appear in other forms—for instance, one which requires 0.035 millimicrograms of B<sub>12</sub> is quite common, as also are variants which require 0.018 millimicrograms of B<sub>12</sub>. The interesting thing is that this B<sub>12</sub> requirement can be substituted completely in the latter case by traces of homocysteine; and this implies in the first place that there is a lack of a sulfhydryl in B<sub>12</sub> deficiency, because the oxidized form will not substitute. The interesting thing is that only catalytic amounts are required so it seems that homocysteine does not act simply by furnishing the methionine skeleton. The mutant which requires 0.035 millimicrograms of vitamin B<sub>12</sub> requires more homocysteine and has to be supplemented with dimethyl-B-propiothetin or S-methyl adenosine and, for maximum growth, para-amino benzoic acid. The original mutant which requires 0.012 millimicrograms of vitamin B<sub>12</sub> will hardly grow at all on any combination of these compounds. I think it should be pointed out that, despite the fact that we apparently have the components required for methionine synthesis, that is, the usual methyl acceptor and donor, there is no evidence whatever from enzymatic experiments that the reaction goes this way at all. It may be that the mechanism of methionine synthesis in microorganisms may not involve the usual transmethylation reactions found in mammals. The other point is that we have to find some role for dimethyl thetins both in animals and in microorganisms. These compounds are highly specific and very effective. Their true role in the organism as sources of methyl groups or as catalysts for transfer is unknown.

DR. HANDLER: There are several comments I would like to make with respect to Dr. Singer's admirable review. He mentioned the discrepancy between our figure for the optimal pH for sulfite oxidation (9.5) and his (7.5). Our conditions were quite dissimilar, however, as Dr. Singer has told me that he always had methylene blue in his reaction mixtures, whereas we have not done so when O<sub>2</sub> consumption in the Warburg was used as an index of activity. That this is a material difference has become obvious with the observation by Mr. Fridovich, in our laboratory, that dialysis of a crude sulfite oxidase preparation from rat liver is without effect when O<sub>2</sub> consumption is measured but inactivated the system with respect to methylene blue reduction by sulfite. Activity is completely restored by addition of liver 'kochsaft' and this has been traced to the presence of an organic cofactor which seems unrelated to known materials but has been partially purified by Dowex chromatography.

The situation with respect to purification of the enzyme still holds; we consistently obtain two ammonium sulfate fractions [at low and high concentrations] which together are considerably more than additive in their activity. Both are active alone but the middle cut, between them, is devoid



of activity. The high fraction appears to be a metallo-flavoprotein whose metal is, as yet, unidentified, and which will reduce methylene blue or cytochrome C in the presence of sulfite while, respiring in the presence of  $O_2$ , and without added cytochromes, it generates  $H_2O_2$ .

We suspect that this may be the real sulfite oxidase. The role of the other protein fraction and the methylene blue reducing systems are currently being studied further but we have the feeling that here we may be watching the effect of  $SO_3^-$  in a system whose normal functioning may be quite unrelated to this metabolite.

In the course of these studies we have encountered and partially purified an enzyme from animal liver and kidney which catalyzes a surprising reaction, namely the desmolysis of  $\alpha$ -hydroxy sulfonic acids (carbonyl—bisulfite addition compounds) to the free aldehyde or ketone and bisulfite. The presence of such an enzyme is certainly surprising and we can only speculate that it serves to break up such compounds as they may form between the diverse carbonyl compounds arising in metabolism and  $SO_2$  formed in the desulfination of pyruvyl sulfinic acid.

Finally, we are also among those who have unsuccessfully sought the pathway of sulfur fixation. The notion of reversing the cysteine sulfinic acid path appeared attractive and Dr. Cobey found that *E. coli*, under strictly anaerobic conditions, could use  $SO_4^{=}$ ,  $SO_3^-$ , and cysteine sulfinic acid equally well for growth while cysteic acid was useless and taurine little better. However, when cysteine sulfinic acid was incubated with various preparations from *E. coli* (or rabbit liver) together with  $S^{32}O_3^-$  and ATP, malate, pyruvate, lactate, glutamate and phosphopyruvate in various admixtures, the cysteine sulfinic acid disappeared as expected and, if the reaction was stopped when about  $\frac{2}{3}$  over, no radioactivity could be found in the residual cysteine sulfinic acid.

Part V

*METABOLISM OF GLYCINE AND SERINE*





# THE SYNTHESIS AND DEGRADATION OF GLYCINE<sup>1</sup>

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GLYCINE IS AN amino acid whose biochemical versatility is being increasingly appreciated. Early investigators, confronted with many examples of its unconventional metabolic behavior, were inclined to regard glycine as the black sheep of the amino acid family. Though it was known to be formed and destroyed in the body in virtually unlimited quantities, the mechanisms of its synthesis and degradation did not seem to follow accepted patterns, nor did it appear to participate in many of the reactions common to other amino acids. As the mystery surrounding the metabolism of glycine is gradually being dispelled (principally through the application of the isotope tracer technique), this amino acid is being revealed as an extraordinarily active substance, participating in many activities of the cell over and above its passive role as a protein constituent. It is a building block for several fundamentally important cell constituents, and is either an end-product or a precursor of substances which enter into diverse metabolic activities.

Fig. 1 gives an indication of the wide range of metabolic channels through which glycine is routed. It can be incorporated into protein and glutathione, or conjugated with bile acids, or with aromatic acids such as benzoic acid; it can be converted to serine, and thence into channels of carbohydrate and fatty acid metabolism via pyruvate and acetate; it can be deaminated to glyoxylate; it can undergo transamidination to glycocyanine, which in turn is converted to creatine.

<sup>1</sup> The experimental work reported here was carried out in collaboration with Miss Bernice Friedmann and Dr. Henry Nakada, and was supported by grants from The National Cancer Institute of the Department of Health, Education and Welfare, the Atomic Energy Commission (contract no. AT(30-1)777), and the American Cancer Society.



It is built into the porphyrins and into the purines. Nearly all of these paths represent mechanisms by which the carbons and nitrogen of glycine may be degraded or excreted. At the same time glycine may be formed from carbohydrate, either via serine or possibly via glycolaldehyde, glycolate, and glyoxylate (the latter path being as yet hypothetical). It is formed from threonine. It may also be

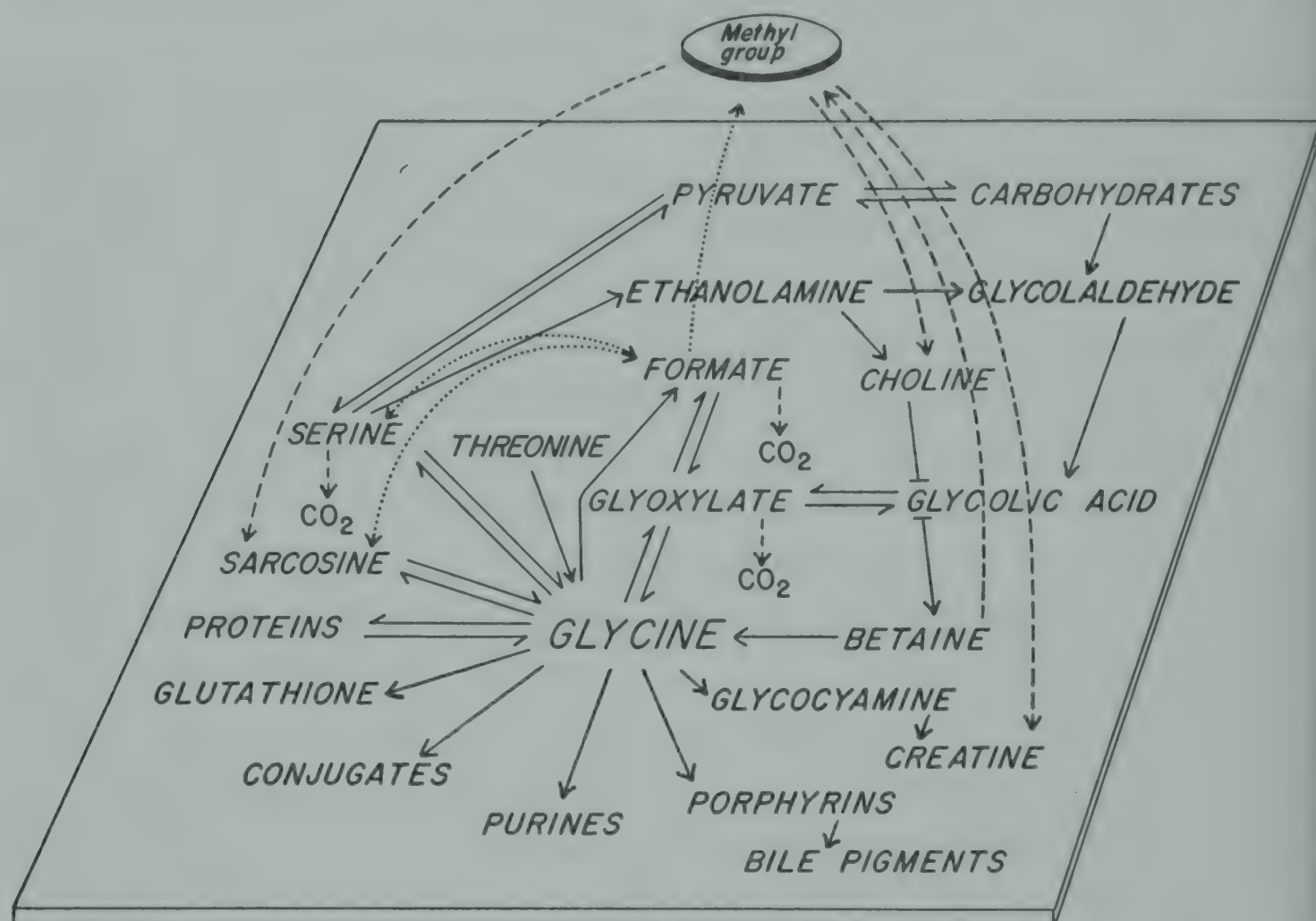


FIG. 1.

formed from serine indirectly via ethanolamine and glycolaldehyde, or again indirectly from serine via ethanolamine, choline, and betaine. Associated with all of these more or less direct transformations, arranged on the horizontal plane, there is a bewildering variety of transfers of methyl groups, or of other one-carbon fragments here designated rather inaccurately as formate.

To carry out the assigned topic of discussing the synthesis and degradation of glycine, completely and exhaustively, would extend this discussion into many fields which are already covered by other speakers in this symposium. I therefore restrict this discussion to one

metabolic pathway, one which studies conducted in our laboratory indicate may be a major route of glycine catabolism in the rat and which may play a role in glycine synthesis in diverse organisms. This route involves the direct interconversion of glycine and glyoxylic acid.

Because of their close chemical relationship, it is logical to consider that glyoxylic and glycolic acids and glycine are also related metabolically. Despite many efforts in the past, however, evidence bearing on the interconversions of these substances has been indecisive and contradictory (1, 2).

Our interest in this possibility was stimulated by several recent developments. The early labeling of glycolic acid and glycine during photosynthesis with  $C^{14}O_2$  (3) indicates a close metabolic relationship between these two substances in green plants. Glycolaldehyde, a plausible precursor of glyoxylate, appears to be a product of sugar catabolism (4). The possibility existed at the time this work was undertaken that oxalate might be an intermediate of carbohydrate metabolism in certain organisms (5, 6) and that it might be reduced to glyoxylate. Finally, the possibility that acetic acid might undergo direct oxidation to glycolate or glyoxylate (7) deserved consideration.

To obtain direct information concerning the role of these substances as glycine precursors, Miss Friedmann and I (8) conducted a series of experiments in which isotopically labeled compounds were administered to rats together with a dose of sodium benzoate, following which hippuric acid was isolated from the urine and assayed for radioactivity. At the same time, oxalic acid isolated from the urine with the aid of carrier, and respiratory  $CO_2$ , were also assayed. The results of these experiments are summarized in Table 1.

The most striking observation was the rapidity with which glyoxylate and glycolate were converted to glycine in the rat. From these data it is evident that glycolate is about as good a source of hippuric acid glycine as glycine itself, and glyoxylate appears to be even a better source than glycine. This curious result, which has been confirmed subsequently by Chao et al. (9) and by Weissbach and



TABLE 1

CONVERSION OF VARIOUS ACIDS TO GLYCINE, OXALIC ACID, AND  
CO<sub>2</sub> IN THE INTACT RAT

Values are in per cent of radioactivity injected.

Compound Injected	PRODUCTS		
	Glycine %	Oxalic Acid %	Carbon Dioxide %
Glyoxylic acid-1,2-C <sup>14</sup>	22.0	27.1	16
Glycolic acid-2-C <sup>14</sup>	7.2	0.8	32
Glycolic acid-1-C <sup>14</sup>	11.4	1.1	13
Glycine-2-C <sup>14</sup>	12.5	0	13
Glycine-1-C <sup>14</sup>	13.1	0.2	27
Oxalic acid-1,2-C <sup>14</sup>	0	18.5	1
Formic acid-C <sup>14</sup>	0	0	97
Acetic acid-2-C <sup>14</sup>	0	0.4	55

Sprinson (10), is to be regarded more as a reflection on the quantitative reliability of the method than as an indication that glycolate and glyoxylate are more direct precursors of hippuric acid glycine than glycine itself. It is probably due to differences in absorption rate, or to dilution by endogenous glycine, or to other factors which remain obscure in an intact animal. Nevertheless, the results demonstrate that conversion of glycolate and glyoxylate to glycine is remarkably rapid in vivo. The fact that the rates of conversion are similar for both glycolate carbons indicates that it occurs directly, without alteration in the carbon chain, presumably with glyoxylate as the direct source of glycine.

It is now well established, as a result of experiments by Sakami (11) and others (12), that formate is produced metabolically from the glycine  $\alpha$ -carbon. The results of the present study indicated that this process is not appreciably reversible. Though injected formate was readily oxidized by the intact rat, in the experiment shown in Table 1, 97 per cent having been oxidized within 6 hours after administration, there was no appreciable appearance of formate carbon in hippuric acid. In similar fashion we established that acetate was not incorporated into glycine in an appreciable amount. The fact that acetate was readily oxidized, 55 per cent of the isotopic

carbon having been recovered as  $\text{CO}_2$  within 6 hours after injection, and without any appreciable conversion to glycine, is regarded as good evidence against its direct oxidation to glycolate or glyoxylate in the rat.

This study is of further interest for the light it sheds on oxalic acid metabolism. The data here presented leave no doubt that oxalic acid is metabolically inert in the intact rat. It is not oxidized to  $\text{CO}_2$ , and is evidently not reduced to glyoxylate, since its carbon was not found in hippuric acid. Oxalic acid is formed readily, however, from glyoxylic acid (cf. 10), in the experiments shown in Table 1 27 per cent of the injected radioactive carbon having been recovered as oxalic acid. In contrast, neither glycolic acid nor glycine yielded appreciable quantities of oxalate. We shall return to this apparently anomalous observation later.

TABLE 2

## A SURVEY OF GLYCINE OXIDATION IN VARIOUS TISSUES OF THE RAT

Values are given in  $\mu$ atoms labeled carbon oxidized per hour per g. dry tissue.

Tissue	SUBSTRATE			
	Glycine-1- $\text{C}^{14}$		Glycine-2- $\text{C}^{14}$	
	Slices	Homo- genate	Slices	Homo- genate
Liver	10.2	52.7	2.5	11.0
Kidney	54.4	17.9	8.6	7.4
Heart	0.2	0		0
Spleen	0.2	0		0
Lung				0
Brain	1.1	0		0
Skeletal muscle		0		0
Rhabdomyosarcoma (mouse)		0		0

The speed with which glyoxylic and glycolic acids were converted to glycine, coupled with the fact that glyoxylate was readily oxidized to  $\text{CO}_2$ , encouraged a further investigation of the possibility that the reversal of glycine formation by this process might play a part in the degradation of this amino acid. To assess the occurrence of this mechanism of glycine degradation a study was undertaken with



Mr. Henry Nakada, using isolated surviving rat tissues (13). Results of a survey of the ability of various rat tissues to carry out the oxidation of glycine are shown in Table 2. The values in this table are in microatoms of labeled glycine carbon converted to  $\text{CO}_2$  per gram of dry tissue per hour. Slices or homogenates of liver, kidney, and to a much lesser extent, of brain, oxidized the glycine  $\alpha$  and carboxyl carbons. The rates in liver and in kidney were quite high—they were of a magnitude similar to that of fatty acid oxidation in these tissues. The amino acid was, however, oxidized at a minimal rate or not at all in heart, spleen, lung, and skeletal muscle. Evidently the ability to oxidize glycine is not a general attribute of rat tissues. Subsequent work with in vitro systems was restricted principally to rat liver, a tissue in which glycine oxidation was most regularly active.

TABLE 3

FORMATION OF HIPPURATE, OXALATE, AND  $\text{CO}_2$  IN RAT LIVER SLICES

Substrates in 0.01 *M.* concentration together with 0.01 *M.* sodium benzoate. Incubations were carried out for 2 hrs. at 37° C. with oxygen in the gas phase. Values are in  $\mu\text{atoms C/g. dry tissue/hr.}$

Substrate	$\text{Q}_{\text{O}_2}$	PRODUCTS		
		Respiratory $\text{CO}_2$	Hippuric acid	Oxalic acid
Glycine-2- $\text{C}^{14}$	8.25	8.82	23.5	0
Glycolate-2- $\text{C}^{14}$	7.86	10.1	15.1	19.6
Glyoxylate-1,2- $\text{C}^{14}$	5.65	19.6	11.1	30.0

In the experiment shown in Table 3, glycine, glycolate, and glyoxylate, all labeled with carbon 14, were metabolized by liver slices in oxygen, and their respective conversions to carbon dioxide, to hippuric acid, and to oxalic acid were followed.

All three acids studied were oxidized to  $\text{CO}_2$  in liver slices, and a relatively high rate of glyoxylate oxidation as compared with that of glycine was in accord with the idea that glyoxylate may be on the pathway of glycine oxidation in liver slices. As in the intact animal, all three substances were converted to hippuric acid. Again as in the intact rat, the paradoxical result was observed that, whereas

glyoxylate and glycolate yielded large amounts of oxalate, essentially no oxalate formation was observed from glycine. This raised serious doubts whether glycine is converted metabolically to glyoxylate. However, a specific test of this question by an isotope-trapping procedure revealed that in liver homogenate glycine is indeed transformed to glyoxylate. Typical results of such experiments are shown in Table 4.

TABLE 4

## CONVERSION OF GLYCINE TO GLYOXYLIC ACID

Values are given in  $\mu$ atoms of glycine carbon converted/g. dry tissue/hr. Glycine-2- $C^{14}$ , 0.02 M.; glyoxylate, 0.01 M. Incubation proceeded for 2 hrs. at 37° C. in air as gas phase with 5 g. rat liver homogenate suspended in 25 ml. KCl— $PO_4$  solution, pH 7.8.

Product	Assayed as	Glycine Carbon $\mu$ atoms
Respiratory $CO_2$	Such	1.12
Glyoxylate-2-4-dinitrophenylhydrazone	Such	10.45
Glyoxylate $\alpha$ -carbon	Acetate methyl <sup>a</sup>	9.23
Glyoxylate COOH carbon	Acetate COOH <sup>a</sup>	0
Oxalic acid		1.39

<sup>a</sup> Counted as  $BaCO_3$ .

In this experiment glycine-2- $C^{14}$  was incubated in air with a whole homogenate of rat liver, together with a pool of unlabeled glyoxylic acid. After two hours' incubation, the remaining glyoxylate was isolated and purified as the 2,4-dinitrophenylhydrazone. Respiratory  $CO_2$  was collected in this experiment, and oxalate was isolated with the aid of carrier. A large amount of the activity originally present in glycine appeared in the hydrazone of glyoxylic acid. This result provided unequivocal evidence of a conversion of glycine to glyoxylate. The fact that the activity trapped in glyoxylate was of similar magnitude to that ordinarily converted to  $CO_2$  in this type of experiment, coupled with the observation that only a small quantity of radioactivity appeared in the respiratory  $CO_2$  in the presence of the glyoxylate pool, suggested that glyoxylate is an intermediate stage on the pathway of glycine oxidation. That this transformation was direct is indicated by the presence of essentially all of the glycine



$\alpha$ -carbon in the  $\alpha$ -carbon of glyoxylate. The distribution was established by reducing the hydrazone to acetic acid, and decarboxylating the latter by means of hydrazoic acid.

A clue to the curious lack of oxalate formation from glycine was obtained when it was found that in this experiment an appreciable quantity of the glycine  $\alpha$ -carbon was converted to oxalic acid. It appeared from these results that glyoxylate may yield oxalate only when present in relatively high concentration, but not when it is formed transiently and is present in low concentration. The data of the experiment shown in Table 5 demonstrate the essential correct-

TABLE 5

EFFECT OF SUBSTRATE CONCENTRATION ON OXALATE FORMATION FROM GLYOXYLATE IN WHOLE LIVER HOMOGENATE

Values are in  $\mu$ atoms glyoxylate carbon converted/hr./g. dry tissue.

Glyoxylate concentration M.	RAT LIVER HOMOGENATE			PIGEON LIVER HOMOGENATE		
	Total O <sub>2</sub> uptake $\mu$ l.	CO <sub>2</sub> $\mu$ atoms C	Oxalate $\mu$ atoms C	Total O <sub>2</sub> uptake $\mu$ l.	CO <sub>2</sub> $\mu$ atoms C	Oxalate $\mu$ atoms C
0.0005				465	7.76	0
0.001	3020	7.4	0	384	11.8	1.1
0.005	2420	26.3	10.5	346	30.0	13.4
0.010	1310	25.3	17.5	187	24.4	43.5

ness of this hypothesis. As shown in columns 4 and 7, glyoxylate in a concentration of 0.001 M. or below undergoes oxidation to CO<sub>2</sub>, but yields little or no oxalate. At higher concentrations, oxalate formation increased markedly, and at 0.01 M., oxalate formation was almost as high as CO<sub>2</sub> production. It is evident that glyoxylate, when formed as a metabolic intermediate, and is present in low concentration, need not necessarily produce oxalate; the non-formation of oxalate from glycine carbon therefore need not weigh against consideration of glyoxylate as an intermediate of glycine oxidation.

Further efforts in these experiments were directed to discover how glyoxylate is converted to CO<sub>2</sub>. It was already evident that oxalate was not an intermediate in this process; the most likely possibility appeared to be a direct oxidation to formic acid and CO<sub>2</sub>, with

subsequent oxidation of formate to  $\text{CO}_2$ . To assess this possibility, the production of formate from glycolate and glyoxylate was compared with that from glycine, a conversion already demonstrated *in vitro* by Sakami (11) and by Greenberg and his coworkers (12). Since formate does not ordinarily accumulate in these experiments, its production was ascertained by trapping metabolic formate from labeled precursors, using the usual procedure of adding a pool of unlabeled formate to the tissue preparation. Results of such experiments are shown in Table 6. As anticipated, no labeled

TABLE 6

PRODUCTION OF FORMATE FROM GLYCINE, GLYCOLATE, AND GLYOXYLATE IN RAT LIVER PREPARATIONS

Values are given in  $\mu$ atoms of labeled substrate carbon converted/hr./g. dry tissue.

Substrate	$\text{CO}_2$	Slices Formate	Whole Homogenate		Washed Particles	
			$\text{CO}_2$	Formate	$\text{CO}_2$	Formate
Glycine-1- $\text{C}^{14}$	4.9	0	—	—	—	—
Glycine-2- $\text{C}^{14}$	2.1	3.76	19.5	5.1	3.08	1.45
Glycolate-1- $\text{C}^{14}$	9.8	0	—	—	—	—
Glycolate-2- $\text{C}^{14}$	7.5	3.03	2.17	2.6	1.38	8.65
Glyoxylate-1,2- $\text{C}^{14}$	26.8	10.3	22.8	5.4	3.42	3.28
Oxalate-1,2- $\text{C}^{14}$	0	0	—	—	0	0
Serine-3- $\text{C}^{14}$	—	—	35.8	6.9	—	—

formate arose from the carboxyl carbons of glycine or glycolate, nor was it produced from oxalate. However, formate was produced in large amounts from the  $\alpha$ -carbons of glycine and glycolate, and its production was particularly high with labeled glyoxylate as substrate. Formate production in a relatively large amount was observed from these substrates in slices, in whole homogenates, and in the washed insoluble particles separated from the whole homogenate by high-speed centrifugation. These findings suggested that in liver, at least, formate is a major intermediary metabolite of glycine. The high rate of formate production from glyoxylate is in keeping with the idea that glyoxylate may be an intermediate in formate production from glycine. Finally, it was shown that formate was rapidly



oxidized to  $\text{CO}_2$  in rat tissues generally (Table 7). The particularly high rate in liver and kidney was of special interest, in view of the high rate of glycine oxidation in these two tissues.

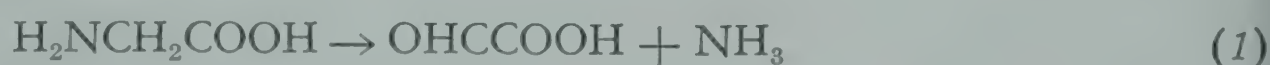
TABLE 7

## A SURVEY OF FORMIC OXIDASE ACTIVITY IN VARIOUS TISSUE HOMOGENATES

Whole tissue homogenates, 330 mg. per vessel, were incubated for 1 hr. at  $37^\circ \text{C}$ . Formate concentration was 0.01 *M*. Values are in  $\mu$ atoms of formate carbon oxidized/g. dry tissue/hr.

Tissue	$\text{CO}_2$ $\mu$ atoms C
Liver	48.3
Kidney	22.7
Heart	7.4
Brain	7.9
Spleen	7.6
Lung	9.1
Muscle	6.9
Rhabdomyosarcoma (mouse)	5.7
Hepatoma 98/15 (mouse)	5.7

The results of these studies *in vitro* thus establish a clear pathway of glycine oxidation in rat liver which may be formulated in the following equations.



It may be of interest now to consider very briefly some of the enzymatic mechanisms involved in these transformations. Two mechanisms are possible for step one: the interconversion of glycine and glyoxylate. Ratner et al. (14) have described a flavoprotein enzyme, glycine oxidase, which catalyzes the oxidative deamination of glycine to glyoxylate. It also acts on sarcosine to yield glyoxylic acid and methylamine. The physiological significance of this enzyme may be questioned, however, because its activity is extremely low

at physiological pH's, and requires for appreciable activity extremely high concentrations of glycine. Up to a few years ago it was believed that glycine did not participate in transamination reactions (15). The rapid interconversions of these substances, observed in our experiments, both in vivo and in vitro, suggested that transamination may have been involved, rather than oxidative deamination and its reversal. The failure of previous investigators to demonstrate transamination with glycine and various keto acids, we reasoned, could be due to an unfavorable equilibrium from the glycine side. Accordingly, we studied transamination with this system, starting with glyoxylate (16). As we anticipated, we found that glycine formation from glyoxylate and glutamate was quite rapid. To our surprise we found that an enzyme was not even needed for this reaction. At room temperature, and at pH 7.4, glyoxylate, when incubated with several amino acids, yielded glycine.

In these experiments, glyoxylate was incubated with various amino acids at room temperature, and samples were tested for the presence of glycine, using paper chromatography. Glycine formation was observed with glutamic acid, glutamine, alanine, arginine, asparagine, aspartic acid, ornithine, and tryptophan. This reaction appeared to be specific for glyoxylate and essentially non-reversible, since neither  $\alpha$ -ketoglutarate nor pyruvate underwent transamination when incubated with glycine or other amino acids. The reaction was considered to involve direct transamination between glyoxylate and the amino acid, since ammonia was inactive, and no glycine was ever detectable with substances other than amino acids. We were somewhat surprised to find that pyridoxamine was not active with glyoxylate, in view of the involvement of the pyridoxamine system in enzymatic transaminations.

There is also an enzyme-catalyzed transamination of glyoxylate, which is considerably more rapid than the non-enzymatic reaction. Table 8 shows the relative magnitude of the two processes in rat liver. With glyoxylate and glutamine alone, or in the presence of inactivated tissue, about 0.6 micromole of glycine was formed under the conditions used. In the presence of liver homogenate, without



TABLE 8

COMPARISON BETWEEN ENZYMATIC AND NON-ENZYMATIC GLYCINE SYNTHESIS  
BY TRANSAMINATION WITH GLYOXYLIC ACID AND GLUTAMINE

The system contained substrates in the concentrations indicated plus 1 ml. of rat liver whole homogenate (prepared by homogenizing 1 g. of liver in 6 ml. of isotonic KCl solution), in a total volume of 3 ml. of phosphate-buffered isotonic KCl solution. The vessels were incubated in air at 37° C. for 4 hours.

Tissue	Glyoxylic acid 0.01 M.	Glutamine 0.02 M.	Glycine Formed	
			Experiment 1 $\mu$ M.	Experiment 2 $\mu$ M.
None	+	0	0	0
None	+	+	0.63	0.675
Rat liver homogenate	0	0	0.94	0.90
Rat liver homogenate	+	0	2.32	2.16
Rat liver homogenate	+	+	7.44	7.95
Boiled rat liver homogenate	+	0	0.54	*
Boiled rat liver homogenate	+	+	0.81	*

\* Not run.

any substrate, 0.9 micromole was found, but the addition of glyoxylate increased the yield of glycine to somewhat more than 2 micromoles. The addition of glyoxylate and glutamine to liver homogenate increased the yield still further, to almost 8 micromoles. Thus the enzyme-catalyzed reaction was about ten times as rapid as the reaction in the absence of tissue, or in the presence of inactivated tissue. Metzler, Olivard, and Snell (17) independently studied this non-enzymatic transamination in much greater detail, using, however, elevated temperatures. They found the reaction is catalyzed by aluminium, cupric, and ferric ions, and the equilibrium is far toward glycine formation. These investigators also found, under conditions of their experiments, that pyridoxamine undergoes transamination with glyoxylate. Our failure to observe transamination non-enzymatically with pyridoxamine is probably due to the use of a lower temperature, and failure to recognize the powerful metal-ion catalysis of this reaction.

While our studies were in progress, and since their completion, a host of other studies have appeared which now leave no doubt

of the ability of the glyoxylate-glycine system to participate in transamination. Cammarata and Cohen (18), using improved methods of measuring transaminase activity, found an appreciable exchange between glycine and  $\alpha$ -ketoglutarate in rat liver extracts, but not in heart or kidney. Meister et al. (19) also observed a rapid transamination between glyoxylate and aspartic acid, asparagine, glutamic acid, and glutamine in transaminase preparations from rat liver. King et al. (20) found extracts from plant seedlings to be quite active in transaminations between glycine and  $\alpha$ -ketoglutarate.

Step 2, the conversion of glyoxylate to formate and  $\text{CO}_2$ , is still not well characterized. Zelitch and Ochoa (21) recently reported that glyoxylic acid is oxidized non-enzymatically to formate and  $\text{CO}_2$  by hydrogen peroxide. Although this non-enzymatic reaction is extremely rapid, it is not yet certain whether there is also an enzyme-catalyzed oxidation of glyoxylate to formate and  $\text{CO}_2$ .

Ratner et al. (14) have shown that xanthine dehydrogenase can oxidize glyoxylate to oxalate (step 2a). In confirmation of these results, we also found that glyoxylate is oxidized to oxalate by a purified xanthine dehydrogenase prepared from cream according to the procedure of Ball (22). However, this is probably not the only enzyme responsible for oxidation of glyoxalate to oxalate in liver, since, as shown in Table 5, glyoxylate was converted to oxalate in homogenates of pigeon liver, a tissue which has been repeatedly reported to contain no xanthine dehydrogenase (23). Evidently, in the rat, conversion of glycine to oxalic acid does not occur to a large extent physiologically. It would appear from the data here presented that oxalate is formed in quantity only when glyoxalate levels are high. With the transamination equilibrium being toward glycine formation, with mechanisms available for reduction of glyoxylate to glycolate (16), and with a pathway for its rapid oxidation to formate and  $\text{CO}_2$ , it seems unlikely that under normal conditions this transient intermediate would accumulate in sufficiently large quantities to yield oxalate. The oxalate excreted regularly in small quantities by animals probably represents in the main dietary oxalate of plant origin. However, the fact that glycolic acid is an early intermediate of photo-



synthesis (3) suggests that this substance may be the precursor of the often observed large accumulations of oxalate in plants.

Step 3, the oxidation of formate, which completes this pathway of glycine degradation, occurs readily in the intact animal; as already shown, it occurs in a wide variety of rat tissues. There are good grounds for the belief that catalase plays a part in this process. Britton Chance (24) has shown that formate is one of a number of substrates which can be dehydrogenated by a catalase-hydrogen-peroxide complex. In support of this idea, we found a marked parallelism between formate oxidation and catalase activity in a wide variety of tissue types, including many neoplastic tissues. Matthews and Vennesland (25) have described an ATP-activated formic oxidase in animal tissues. We believe this may be a coupled oxidation phenomenon. We found the system of Matthews and Vennesland to be activated not only by ATP but also by a variety of purines, particularly hypoxanthine. This leads us to the belief that purine oxidation by xanthine dehydrogenase activates this system by yielding hydrogen peroxide, which, in the presence of catalase, oxidizes formic acid. As shown in Table 9, a model system can be set up, containing catalase and xanthine dehydrogenase, which will oxidize formate only when hypoxanthine is present.

TABLE 9

## OXIDATION OF FORMATE IN MODEL SYSTEM

Each vessel contained 1 ml. xanthine oxidase, 0.1 ml. catalase, and substrates and buffer to 3 ml. Vessels incubated for 1 hr. in air at 37° C.

Xanthine Oxidase	Catalase	Formate-C <sup>14</sup> (0.01 M.)	Hypo-xanthine (0.001 M.)	Oxygen Uptake	Total Counts <sup>a</sup> in CO <sub>2</sub>
+	—	+	+	95	22000
+	+	+	+	158	40000
+	—	+	—	0	0

<sup>a</sup> Total Counts = specific activity X  $\mu$ moles of BaCO<sub>3</sub>.

This direct pathway of glycine degradation having been established in rat liver, it was of interest to compare its quantitative

significance with the one which has hitherto been considered to play a major role in glycine oxidation, namely, the pathway via serine, pyruvate, acetate, and the citric acid cycle. The establishment of this means of glycine oxidation stems from the now well-established rapid interconversion of glycine and serine, to be discussed by Sakami; and the conversion of serine to pyruvate, demonstrated by Chargaff and Sprinson (2) to occur in various bacteria and in extracts of mouse, rat, and rabbit liver. To obtain information concerning the relative quantitative importance of these two pathways, a number of experiments, as yet unpublished, were conducted by Dr. Nakada with labeled substrates, in the presence and the absence of supposed intermediates.

As shown in Table 10, glycine, glyoxylate, and serine, when present separately, were oxidized readily, as shown by the high conversion coefficients. The presence of serine and glyoxylate greatly lowered glycine oxidation, and the presence of glycine and glyoxylate also lowered serine oxidation. However, glycine and serine had no effect on glyoxylate oxidation. Though interpretation of such experiments is not simple, they are in accord with the idea that glyoxylate represents an intermediate in glycine oxidation and possibly also in serine oxidation, but that neither glycine nor serine is an intermediate in glyoxylate oxidation.

TABLE 10

OXIDATION OF GLYCINE, GLYOXYLATE, AND SERINE  
BY RAT LIVER HOMOGENATES

Substrates, in 0.01 *M.* concentration, were incubated one hour at 37° C. with air in the gas phase in a suspension of 330 mg. fresh wt. tissue in a total volume of 3 ml. Values are given in  $\mu$ atoms of carbon converted to CO<sub>2</sub>/hr./g. dry tissue.

Labeled	Additions	Unlabeled	Respiratory CO <sub>2</sub> $\mu$ atoms C
Glycine-2-C <sup>14</sup>		None	29.0
Serine-3-C <sup>14</sup>		None	26.0
Glyoxylate-1,2-C <sup>14</sup>		None	36.4
Glycine-2-C <sup>14</sup>	Serine + Glyoxylate		4.4
Serine-3-C <sup>14</sup>	Glycine + Glyoxylate		12.8
Glyoxylate-1,2-C <sup>14</sup>	Glycine + Serine		38.2



With this possibility in mind, several other experiments were carried out, using  $C^{14}$ -labeled substrates, experiments which we believe more decisively demonstrate the relative participation of these two pathways. Advantage was taken of the fact that rat liver slices can be made to produce large amounts of acetoacetate by the addition of a short-chain acid such as butyric acid. It was felt that if glycine, serine, or both, are oxidized via pyruvate and acetate, carbon from these substances would mix with the acetyl groups being formed from butyrate, and would be incorporated into acetoacetate. Thus the level of radioactivity in acetoacetate would provide some idea of the quantitative importance of acetate as an intermediary of glycine and serine oxidation. At the same time, formate and serine were recovered by carrier addition and assayed, as was respiratory  $CO_2$ .

TABLE 11

METABOLISM OF  $C^{14}$ -LABELED GLYCINE, SERINE, AND LACTATE  
BY FASTED RAT LIVER SLICES

Values are in  $\mu$ atoms labeled carbon of the substrate converted to the product/hr./g. dry weight of tissue. Each flask contained 3 g. fresh tissue, substrates, and butyrate at 0.01 M. concentration, in a final volume of 30 ml. Flasks were incubated 2 hours at 37° C. with oxygen in the gas phase.

Additions	PRODUCTS ISOLATED			
	$CO_2$	Acetoacetate	Formate	Serine
DL-Lactate-3- $C^{14}$	30.4	60.4	0.8	2.0
DL-Serine-3- $C^{14}$	47.3	2.3	2.1	—
DL-Serine + Sodium Formate	23.0	3.0	22.1	+
Glycine-2- $C^{14}$	13.6	0.9	1.0	16.8
Glycine-2- $C^{14}$ + Sodium Formate	5.9	1.9	11.0	23.0
Glycine-2- $C^{14}$ + DL-Serine	7.3	1.8	1.2	58.2

As seen from Table 11, DL-lactate was readily oxidized to  $CO_2$ . In keeping with its known route of catabolism via pyruvate and acetate, the acetoacetate produced in this experiment was highly labeled; a large amount of lactate  $\beta$ -carbon could be accounted for therein. This was the type of result anticipated by a known pyruvate producer. However, with the same tissue, under exactly the same conditions, serine carbon, though readily oxidized, was converted

to acetoacetate to only a very small extent. It is thus evident that under the conditions of these experiments serine does not yield large numbers of acetyl groups. Though serine did yield some formate (considerably more than did lactate), the amount of serine  $\beta$ -carbon thus accounted for was small in comparison with the amount oxidized to  $\text{CO}_2$ . Since formate is readily oxidized by liver slices, however, it seemed probable that the amounts of formate produced may have been considerably larger than was indicated by these figures. That this was so is shown in the next flask, one in which the labeled serine was accompanied by a pool of unlabeled formate. In this instance large amounts of serine  $\beta$ -carbon were trapped and recovered as formate, and at the same time, correspondingly less of the labeled carbon appeared in the  $\text{CO}_2$ . These findings indicate that formate is a major intermediary in the oxidation of the serine  $\beta$ -carbon in rat liver.

Though its oxidation occurred at a much lower rate, the glycine  $\alpha$ -carbon displayed much the same pattern of catabolism as did the serine  $\beta$ -carbon. It was converted to only a small extent to acetoacetate in comparison with its oxidation to  $\text{CO}_2$ , but was converted readily to formate, as indicated by the results of this trapping experiment. These experiments also clearly indicate that the glycine-serine transformation is quite rapid. This is shown particularly in the last instance, in which the addition of a serine pool resulted in the trapping of a large quantity of glycine  $\alpha$ -carbon as serine.

Without going into many further implications of this experiment, it seems clear that though glycine and serine are freely interconvertible, little glycine carbon or serine carbon is converted to pyruvate or other substances the oxidation of which proceeds via acetate. On the contrary, the results suggest that serine catabolism proceeds, in rat liver at least, via glycine and glyoxylate. We emphasize, however, that the extension of these results to other tissues, or to the whole animal, would be unjustified without further documentation.

It will be appropriate to close with a few remarks concerning the significance of this direct pathway for the synthesis and degradation of glycine in the intact organism. Arnstein and Neuberger (26),



using a method involving long-term feeding of isotopic glycine or serine to rats, calculated that the rate of serine synthesis is sufficiently rapid to supply all of the glycine required by growing rats. After an exhaustive survey of glycine biosynthesis pathways, Arnstein (27)<sup>2</sup> concluded that the main bulk of the glycine produced by animals is derived from serine, which in turn comes from dietary carbohydrate via pyruvate or some other 3-carbon intermediate.

In animals, the direct synthesis under discussion here is in doubt because no means are yet apparent by which common foodstuffs can yield glyoxylate. The possible existence of such pathways is foreshadowed, however, by the fact that glycolaldehyde, in an enzyme-bound form, is a product of the metabolism of pentoses and other sugars (4, 28, 29). Though the transformation of glycolaldehyde to glyoxylic acid has not yet been demonstrated, such a conversion appears plausible. Weissbach and Sprinson (10) have suggested that glycolaldehyde and glyoxalate are successive intermediates in the conversion of ethanolamine to glycine.

In support of this view, we have found in recent experiments conducted by Miss Friedmann that the intact rat converts carbon-1 of ribose to hippuric acid glycine about as rapidly as it does carbon-1 or the other carbons of glucose. Though these experiments do not permit a quantitative evaluation of glycine biosynthesis pathways, they suggest that synthesis via glycolaldehyde arising from carbons-1 and -2 of a pentose may be potentially as rapid as its formation via pyruvate and serine. The recent demonstration by Racker, De la Haba, and Leder (30) that transketolase splits fructose-6-phosphate into glycolaldehyde and tetrose moieties extends the possible scope of glycolaldehyde metabolism in animal tissues by demonstrating how glycolaldehyde may be formed directly from such an abundant intermediate of glucose catabolism as fructose-6-phosphate.

It is probable that in the green plant and in certain microorganisms glyoxylate represents an important gateway to glycine synthesis. The studies of Calvin and associates (3) have shown that glycolic

<sup>2</sup> I am greatly indebted to Dr. Arnstein for allowing me to see his manuscript before publication.



acid and glycine rapidly incorporate  $\text{CO}_2$  during photosynthesis, the former probably being closely related chemically to a  $\text{CO}_2$  acceptor. Glycolate is a metabolic product in certain fungi (5); and recently Campbell et al. (31) have reported that enzymes exist in *Pseudomonas aeruginosa* for splitting citrate into succinate and glyoxylate, thus providing a pathway of glycine synthesis via the citric acid cycle.

With regard to the role of this pathway in glycine degradation, the evidence already presented leaves little doubt that a substantial portion of glycine is metabolized via glyoxylate and formate in rat liver in vitro. However, we are not yet certain to what extent this occurs in the whole animal. In fact, the following observations can be cited against that occurrence. Although there is an extensive production of formate from glycine, glyoxylate, and glycolate in vitro, these substances do not yield much formate in vivo (32). This observation does not support the idea that formate is a major intermediary metabolite of these substances. However, one can argue that an active one-carbon intermediary in vitro may be irreversibly split to free formate, whereas in the intact animal it may undergo further metabolic transformations, without yielding formate as such. Experiments with labeled glycine have shown that it is converted to only a small extent to oxalate, whereas glyoxylate yields much oxalate. We reasoned that if glycine were metabolized via glyoxalate in the whole rat, then the injection of unlabeled glyoxylate, together with labeled glycine, should result in the trapping of glycine carbon therein, and subsequent conversion of glyoxylate to oxalate should then yield more highly labeled oxalate than if glyoxylate were not administered. However, in such experiments conducted in our laboratory by Miss Friedmann, glyoxylate did not appreciably increase the yield of labeled oxalate from labeled glycine. Here again, one can argue that the lifetime of glyoxylate in the intact rat may be so short that it does not have sufficient time to become appreciably labeled before undergoing conversion to oxalate or glycine.

Weissbach and Sprinson (10) observed that in the pigeon the carbon of  $\text{C}^{14}$ -labeled glyoxylate was incorporated into uric acid



in a manner characteristic of glycine. These investigators pointed out that if glyoxylate yielded formate directly, a greater proportion of glyoxylate carbon should have appeared in carbons 2 and 8 of uric acid than was observed. Here, one may again invoke the argument that glyoxylate is so rapidly converted to glycine that its administration is tantamount to that of glycine.

Shemin (33), in his pioneering experiment demonstrating the conversion of serine to glycine in the rat, found that the ratio of  $N^{15}$  to  $C^{13}$  in the hippuric acid glycine was the same as that of the administered serine. This result is incompatible with the idea that glycine and glyoxylate are rapidly interconverted. One can argue, of course, that once a glycine molecule is deaminated, the resultant glyoxylate may not be reaminated because of the competition of other reactions. In view of the favorable equilibrium, however, one would expect at least some reamination.

Another paradox concerns the route of glycine catabolism via serine and pyruvate. Our studies *in vitro* suggest that this represents a quantitatively unimportant route of glycine (or serine) catabolism in rat liver. This conclusion is in agreement with the consensus of early balance studies in suggesting that glycine is not an efficient precursor of liver glycogen (2). On the other hand, serine is unquestionably glycogenic in the intact animal (2), and from our present knowledge of its metabolism it is difficult to see how this amino acid can be converted to glucose without going through a 3-carbon intermediary closely related to pyruvate. Perhaps further light will be thrown on this matter by Dr. Sakami.

Adding to the complexity of glycine metabolism is the possibility that intermediates in porphyrin synthesis may also participate as intermediates of glycine catabolism. We will no doubt hear more about this possibility from Dr. Shemin.

The present situation with regard to the synthesis and degradation of glycine seems to be closely analogous to that of other metabolites, including glucose and fatty acids. Several metabolic pathways are known, through enzyme or isotope studies on individual tissues, but relatively little is known of the relative extent to which they partici-



pate in the normal metabolism of the whole animal. An important future goal in biochemistry is an understanding of the factors which direct and regulate the flow of metabolites through available metabolic channels.

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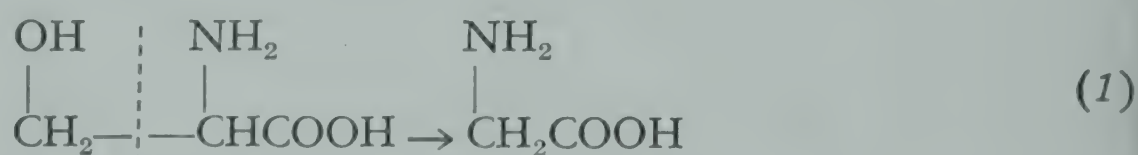


# THE BIOCHEMICAL RELATIONSHIP BETWEEN GLYCINE AND SERINE

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THE EXISTENCE of a biological relationship between glycine and serine was clearly conceived by Knoop (36), who proposed that serine and other  $\beta$ -hydroxy- $\alpha$ -amino acids were converted to glycine by removal of the  $\beta$ -carbon atom.



In support of this concept he presented evidence that  $\beta$ -phenylserine was metabolized via glycine (Fig. 1). The feeding of this amino acid to dogs resulted in the formation of extra benzoic acid which was excreted in the urine as hippuric acid. The presence of mandelic or phenylglyceric acid was not detectable.

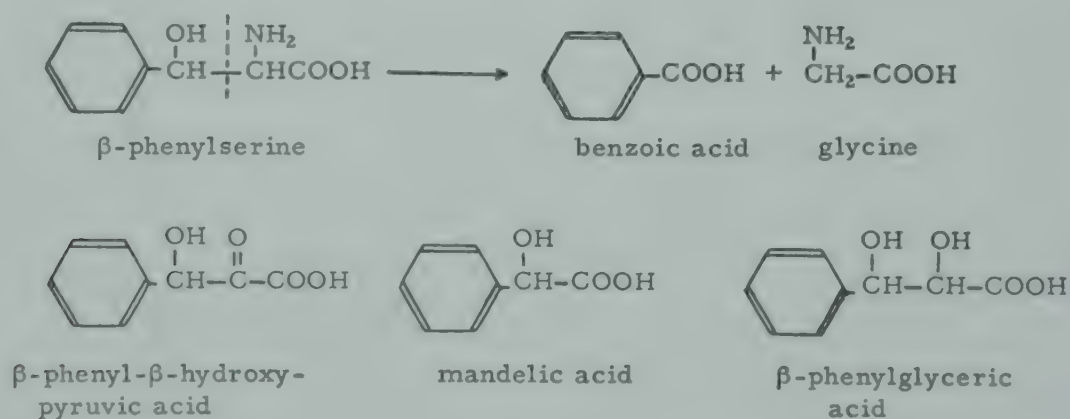


FIG. 1. Metabolism of  $\beta$ -phenylserine.

According to the prevailing belief, amino acids were metabolized to the lower carboxylic acid via the  $\alpha$ -keto acid. Conversion of phenylserine to benzoic acid via  $\beta$ -phenyl- $\beta$ -hydroxypyruvic and mandelic acids, which would be formed by this process, was considered im-

probable, since fed mandelic acid was excreted in the urine with practically no extra benzoic acid. The conversion of phenylserine to benzoic acid via phenylglyceric acid was excluded by a similar argument. These results indicated that phenylserine did not follow the general scheme of amino acid oxidation, and were consistent with a pathway in which glycine was cleaved from the molecule.

Strong support for the existence of a biological interconversion of glycine and serine was first provided by the microbiological studies of Roepke, Libby, and Small (53), who reported the failure of a mutant strain of *E. coli* to grow on their basal medium unless either glycine or serine were provided. The simplest explanation of these findings is that glycine and serine could not be formed from the components of the basal media but could be converted into one another.

Further evidence for the existence of a biological conversion of serine to glycine has been provided by tracer studies. Shemin found that when  $N^{15},1-C^{13}$ -serine was administered to rats and guinea pigs together with benzoic acid, the  $C^{13}$  was incorporated with relatively little dilution into the carboxyl carbon of hippuric-acid glycine (59). The conversion of serine to glycine appeared to have occurred directly with utilization of the carbon chain and nitrogen, since the isolated glycine possessed approximately the same  $N^{15}/C^{13}$  ratio as the administered serine. The reverse process of this conversion has been demonstrated by similar methods. Thus Ehrensvärd et al. (19) showed that the carboxyl- $C^{13}$  of glycine given to *Torulopsis utilis* as a single carbon source was preponderantly transferred to the carboxyl carbon of serine and proline, whereas the carboxyl- $C^{13}$  of alanine was distributed fairly evenly into the carboxyl groups of the various amino acids. Similar results were obtained with cell-free rat liver homogenates by Winnick et al. (73): when 2- $C^{14}$ -glycine was equilibrated with these preparations, 11 to 12 per cent of the  $C^{14}$  incorporated into the protein was present in glycine and 60 per cent in serine. Almost all of the serine activity was in the  $\alpha$ -carbon atom. Utilization of the nitrogen as well as the carbon of glycine in its conversion to serine was indicated by the finding



that the  $N^{15}$  and  $1-C^{13}$  of glycine fed to rats were incorporated into the liver serine with low dilution and without change in  $N^{15}/C^{13}$  ratio (57). Similar results have recently been reported by Elwyn and Sprinson (24).

SOURCES OF THE  $C_1$  UNITS INVOLVED IN THE CONVERSION  
OF GLYCINE TO SERINE, AND PRODUCTS FORMED FROM  
THE SERINE- $\beta$ -CARBON

As a possible source of the " $C_1$ " units involved in the conversion of glycine to serine, formate was first investigated, since the biological occurrence of this substance had been established. It seemed less likely that  $CO_2$  was a precursor of the one-carbon unit, since the incorporation of  $CO_2$  into serine- $\beta$ -carbon followed by the conversion of serine to pyruvate (5, 10) would serve to introduce  $CO_2$ -carbon into the pyruvate methyl group and into the 1,6 and 2,5 carbons of the liver glycogen. These positions were known to acquire very little labeling from  $CO_2-C^{14}$  (26). The inability of  $CO_2$  to form serine- $\beta$ -carbon was later indicated in an experiment in which the isotope concentration of the respiratory  $CO_2$  and serine- $\beta$ -carbon was determined after the administration of  $1-C^{13}$ -glycine (56) (Table 1).

TABLE 1  
LABELING OF THE RESPIRATORY  $CO_2$  AND SERINE AFTER THE  
ADMINISTRATION OF  $1-C^{13}$ -GLYCINE TO RATS (56)

Time (hours)	Labeling of the Respiratory $CO_2$	Time (hours)	Labeling of the Respiratory $CO_2$
	(at. % exs.)		(at. % exs.)
0-1	0.40	8- 9	0.60
2-3	0.85	10-11	0.25
4-5	0.82	13-14	0.12
6-7	0.85		
		(at. % exs.)	
Labeling of the Carbon Atoms of Serine		COOH	$\alpha$
		0.78	$\beta$
			0.00
			0.00

Although  $C^{13}$  was incorporated into the respiratory  $CO_2$  by oxidation of the glycine, it was not introduced into the  $\beta$ -position of the serine. The possibility that rats convert formate to serine- $\beta$ -carbon was demonstrated by the rapid incorporation of formate- $C^{14}$  administered to intact rats into the  $\beta$ -carbon of liver serine (55). Similar results have been obtained by Siekevitz and Greenberg (63) with rat liver slices. Several studies with  $\beta$ - $C^{14}$ -serine have indicated the occurrence of the reverse process, i. e., the formation of formate from serine- $\beta$ -carbon. Elwyn and Sprinson (21) reported that in the pigeon serine- $\beta$ - $C^{14}$  was efficiently incorporated into the ureide carbons of uric acid, which are the positions labeled by formate. Siekevitz and Greenberg (64) observed the formation of formate from serine- $\beta$ -carbon in rat liver slices, and Weinhouse and Friedmann (69) have reported the occurrence of this process in the intact rat.

The possibility that formaldehyde is an intermediate of formate utilization for serine formation was investigated by Siegel and Lafaye. Formaldehyde had been indicated as a normal metabolite by the report of Handler, Bernheim, and Klein (31) that it was formed from dimethylglycine and sarcosine by rat, rabbit, and guinea pig liver particles, and by the implication of these substances as intermediates in the metabolism of choline by Dubnoff (18) and Muntz (45). Siegel and Lafaye found that unfortified rat liver homogenates utilized formaldehyde considerably better than formate for serine- $\beta$ -carbon formation (62). These findings, which have been confirmed by Mitoma and Greenberg (44), were consistent with the possibility that formaldehyde was the " $C_1$ " unit involved in serine biosynthesis. However, Mitoma and Greenberg (44) observed that the addition of a pool of inert formaldehyde to a fortified rat liver homogenate incorporating formate- $C^{14}$  into serine did not reduce the activity of the serine. It appeared that formaldehyde was not an intermediate of formate utilization and that serine synthesis involved neither formate nor formaldehyde per se, but rather an intermediate common to both of these substances.



## THE ROLE OF PYRIDOXAL PHOSPHATE IN SERINE BIOSYNTHESIS

Experimental evidence that pyridoxal phosphate is involved in serine biosynthesis was first provided by Lascelles and Woods (37, see also 38) who demonstrated that pyridoxal was required for maximal synthesis of serine from glycine and formate by washed cells of *Streptococcus faecalis* R deficient in this factor. Pyridoxal was also required for the growth of *Leuconostoc mesenteroides* P 60 when serine was replaced by glycine.

The requirement for pyridoxal phosphate in serine biosynthesis from formate has also been studied in chicks (13). The ability of liver extracts of control and of pyridoxine-deficient chicks, and of deficient chicks that had been treated with desoxypyridoxine, to incorporate formate- $C^{14}$  into serine was tested by incubating these preparations with  $C^{14}$ -formate, glycine, and homocysteine.<sup>1</sup> Serine was isolated by chromatography on Dowex-50 columns. Extracts of control and deficient chicks incorporated equal amounts of formate- $C^{14}$  into serine; however, incubated extracts from deficient chicks that had been injected with desoxypyridoxine contained a significantly lower serine  $C^{14}$  activity (Table 2).

TABLE 2

EFFECT OF PYRIDOXINE DEFICIENCY AND DESOXPYRIDOXINE ON FORMATE INCORPORATION INTO CHICK LIVER EXTRACT (14)

Preparation	Activity of Serine (c. p. m.)
Control	11,610
Deficient	11,890
Deficient, inhibitor-treated	5,600

1 ml. extract, 5  $\mu$ M. formate- $C^{14}$ , 200,000 c. p. m.  
3  $\mu$ M. glycine, 3  $\mu$ M. homocysteine, 2 ml. total volume.  
Incubation 1 hr. under  $N_2$  at 38° C.

<sup>1</sup> White Rock chicks were maintained on starter ration for 4 days (av. wt., 51 g.). One group then received diet #56227 of Reyniers et al. (52). The remainder received this diet with pyridoxine omitted. After 9 days, 4 of the deficient chicks were injected with desoxypyridoxine (16 $\gamma$ ). After 2 hrs. all chicks were sacrificed. The livers of

As shown in Table 3, it was possible partially to restore the activity of deficient, inhibitor-treated preparations by preincubation with pyridoxal phosphate. Inhibition of serine synthesis was also produced by preincubation of liver extracts of deficient chicks with desoxypyridoxine and ATP. Neither desoxypyridoxine nor ATP alone produced any effect. The liver preparation inhibited *in vitro* was

TABLE 3

EFFECT OF PREINCUBATION WITH PYRIDOXAL PHOSPHATE ON FORMATE-C<sup>14</sup> INCORPORATION INTO SERINE IN LIVER EXTRACTS OF PYRIDOXINE-DEFICIENT DESOXYPYRIDOXINE-TREATED CHICKS (14)

Preparation	Activity of Serine (c. p. m.)	
	Serine	Total
Control	10,470	11,630
Deficient, inhibitor-treated	4,870	5,530
Deficient, inhibitor-treated + 600 $\gamma$ pyridoxal phosphate	8,940	9,930

1 ml. extract, 5  $\mu$ M. formate-C<sup>14</sup>, 200,000 c. p. m., 5  $\mu$ M. glycine, 5  $\mu$ M. homocysteine, 2 ml. total volume. Incubation under N<sub>2</sub> at 38° C., 20 min. before and 1 hr. after addition of substrates.

also partially reactivated by incubation with pyridoxal phosphate (Table 4). These experiments in which the inhibition of the conversion of formate carbon to serine has been produced by desoxypyridoxine *in vivo* and *in vitro* implicate pyridoxal phosphate in the conversion of formate to serine in animals. The direct involvement of pyridoxal phosphate in the glycine-“C<sub>1</sub>” condensation process is suggested by the report of Metzler et al. (41, 42) that pyridoxal and alum catalyze a reversible non-enzymatic formation of serine from glycine and formaldehyde. A hypothetical mechanism of this effect of pyridoxal which has been proposed by Metzler et al. (43) applies, with a slight modification, equally well to the biological process (Fig. 2). In this hypothesis, pyridoxal phosphate is considered to

the 6 control chicks (av. wt., 126 g.), 9 deficient chicks (av. wt., 63 g.), and of the 4 deficient chicks that had been injected with desoxypyridoxine (av. wt., 65 g.) were pooled and homogenized with 2 parts of 0.1 M. potassium phosphate buffer, pH 7.4. The homogenates were centrifuged for 30 min. at 60,000  $\times$  g and lyophilized. A 5 per cent solution of these powders was used.



TABLE 4

EFFECT OF PREINCUBATION OF LIVER EXTRACTS OF PYRIDOXINE-DEFICIENT CHICKS WITH DESOXYPYRIDOXINE AND ATP ON FORMATE- $C^{14}$  INCORPORATION INTO SERINE (14)

Additions in First Preincubation	Length of First Preincubation (min.)	Additions in Second Preincubation	Length of Second Preincubation (min.)	Activity of Serine (c. p. m.)
None	20	—	—	10,900
Desoxypyridoxine + ATP	20	—	—	6,600
Desoxypyridoxine + ATP	20	—	20	5,500
Desoxypyridoxine + ATP	20	pyridoxal phosphate	20	8,900

1 ml. extract, 5  $\mu$ M. formate, 200,000 c. p. m., 5  $\mu$ M. glycine, 5  $\mu$ M. homocysteine, 1800 $\gamma$  pyridoxal phosphate, 2  $\mu$ M. desoxypyridoxine, 10  $\mu$ M. ATP, 2 ml. total volume. Incubation under  $N_2$ , 45 min. at 38° C. after addition of substrates.

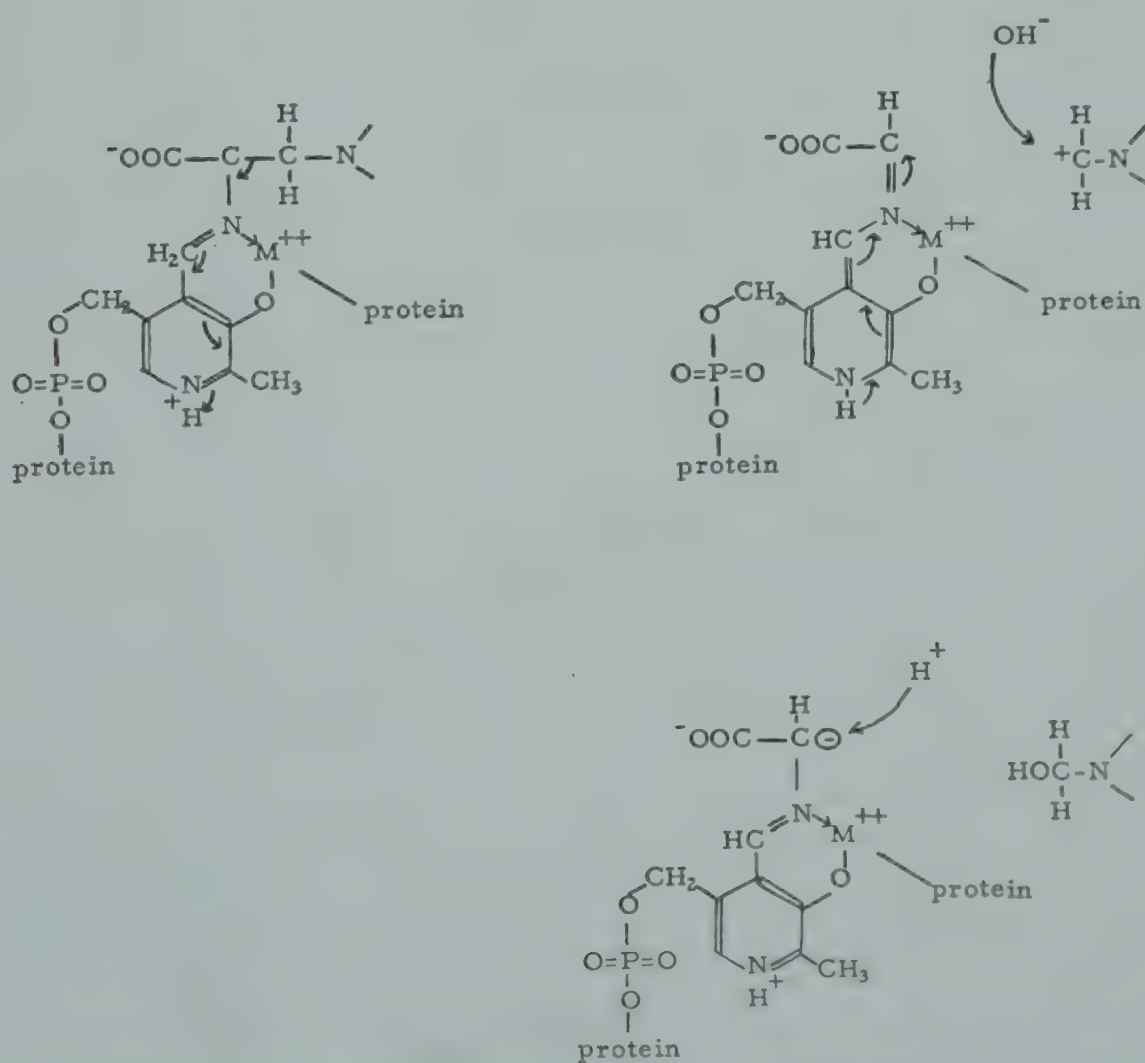


FIG. 2. Proposed mechanism of the biological conversion of serine to glycine. (After Metzler et al., 43).

possess an electron-attracting effect which is transmitted to the  $\alpha$ -carbon of glycine or serine through a Schiff base linkage, displacing the bonds of the amino acids. This displacement would be enhanced by chelation of the complex by a metal ion<sup>2</sup> which would provide a second electron-attracting group. In the conversion of serine to glycine a carbonium cation would be released. The subsequent addition of a proton to the  $\alpha$ -carbon atom followed by hydrolysis of the Schiff base linkage would result in the formation of glycine. The mechanism of the reverse process may be formulated in a similar manner.

Evidence from inhibition studies has been considered to indicate the possibility that pyridoxal phosphate may not be a part of the serine-glycine interconversion enzyme (41). It has been reported that the carbonyl reagents bisulfite (0.005 M.) and *p*-nitrophenylhydrazine (conc. not specified) do not inhibit the conversion of sarcosine methyl groups to serine- $\beta$ -carbon in washed liver particles (44), and that  $\text{NH}_2\text{OH}$  (0.001 M.) does not inhibit the conversion of serine to glycine in liver (67). However, it has also been reported that the transaminases, which are pyridoxal phosphate enzymes, are not inhibited by the carbonyl reagents, semicarbazide (0.002 M.), hydroxylamine (0.002 M.), or phenylhydrazine (0.002 M.) (68).

### THE NATURE OF THE ACTIVE " $\text{C}_1$ " UNIT

The active " $\text{C}_1$ " unit involved in serine biosynthesis appears to be a folic acid derivative at the oxidation level of formaldehyde. The implication of folic acid in the metabolism of compounds containing a single carbon atom was suggested by the identification of the amine accumulating during sulfonamide bacteriostasis of *Escherichia coli* as 4-aminoimidazole-5-carboxamide (60) (Fig. 3). This

<sup>2</sup> Metal ions are required for non-enzymatic pyridoxal-catalyzed reactions but are not established as being a component of pyridoxal phosphate enzymes. It has been suggested that their presence may account for the difficulty in resolving these enzymes into coenzyme and apoenzyme (43). The coordination of the metal ion not occupied by the Schiff base might bind protein moieties, thus firmly linking protein, coenzyme, and substrate. There are, however, other ways in which this binding may occur, such as by attachment of the phosphate group to protein through an ester or amide linkage.



substance also accumulates when *E. coli* is inhibited by aminopterin (75).

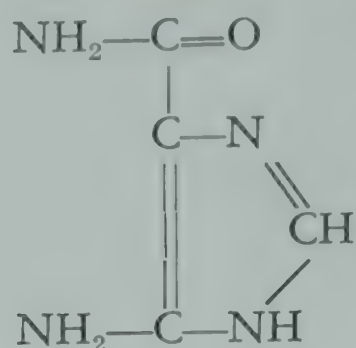


FIG. 3. 4-aminoimidazole-5-carboxamide.

Shive suggested that the carboxamide was converted to purine,<sup>3</sup> a transformation requiring the introduction of a single carbon atom, and further that *p*-aminobenzoic acid or a substance formed from *p*-aminobenzoic acid functioned as a cofactor in this process (60).

The involvement of folic acid in serine biosynthesis has been indicated by numerous investigations. Holland and Meinke (33) showed that a high level of folic acid replaced the serine requirement of *S. faecalis* R for growth. Plaut, Bethel, and Lardy (49) demonstrated that folic-acid-deficient rats incorporate formate-C<sup>14</sup> very poorly into serine-β-carbon (Table 5).

TABLE 5  
FORMATE-C<sup>14</sup> FIXED INTO SERINE OF FOLIC-ACID-TREATED  
AND DEFICIENT RATS (49)

Tissue	Activity of Serine (Net c. p. s. per gm.)	
	Control	Low Folic Acid
Liver	12,300	0
Viscera*	16,500	4,800

\* Kidney, spleen, pancreas, heart, testes, and small intestine.

Totter et al. (66) reported that the conversion of glycine to serine in chick liver homogenates was greatly decreased in folic acid

<sup>3</sup> This transformation does not appear to occur directly. Greenberg (29) has presented evidence indicating that a ribotide of 4-aminoimidazole-5-carboxamide is an intermediate of purine synthesis and that the free carboxamide is a degradation product.

deficiency, and Elwyn and Sprinson (22) demonstrated that the reverse process, the conversion of serine to glycine, was impaired in the deficient rat. Similar results have recently been reported by Braunshtein and Vilenkina (7) and Vilenkina (67), who have indicated that liver preparations of folic-acid-deficient chicks and rats do not synthesize glycine from serine. Lascelles et al. (37, 38) showed that the growth of *L. mesenteroides* P 60 on a serine-free medium in the presence of glycine, CO<sub>2</sub>, and pyridoxal is strongly stimulated by *p*-aminobenzoate, and that cell suspensions of *S. faecalis* R growing on glycine, formate, glucose, and pyridoxal had an almost absolute requirement for folic acid. They also reported that *p*-aminobenzoic acid doubled serine synthesis in a strain of *Saccharomyces cerevisiae* requiring this growth factor.

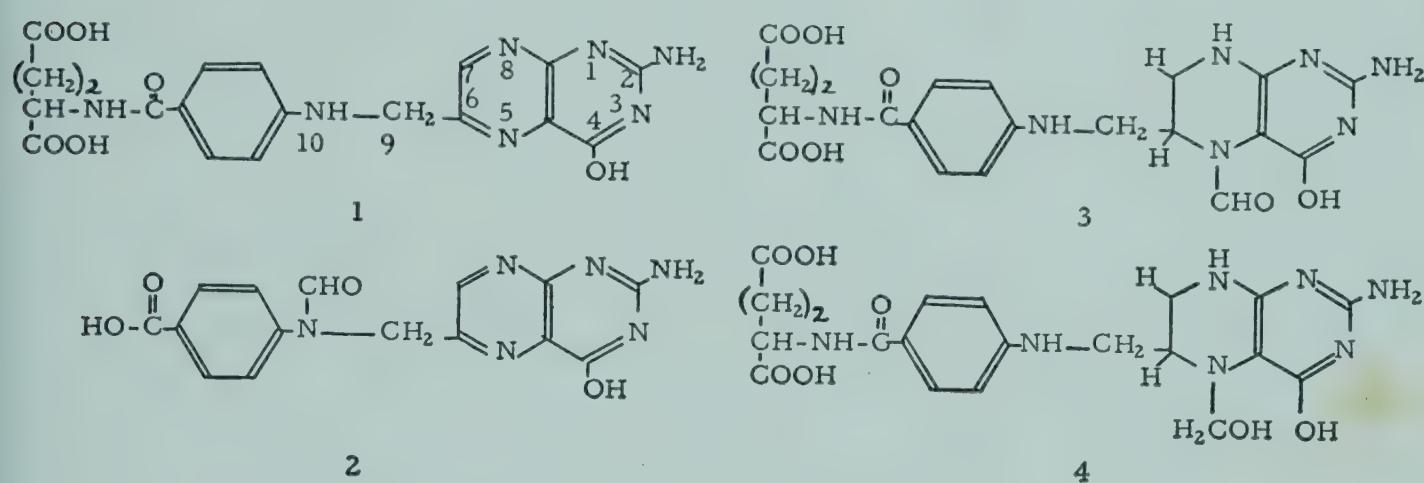


FIG. 4. Structure of Folic Acid and Derivatives

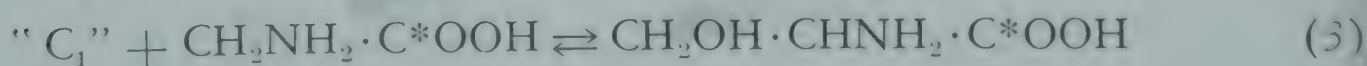
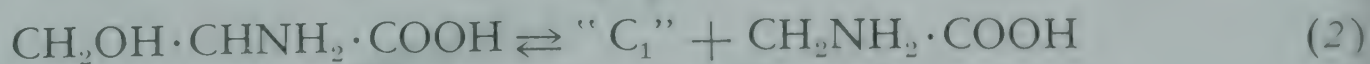
1. Folic acid
2. *Streptococcus lactis* R factor
3. Citrovorum factor (probable structure)
4. 5-Hydroxymethyltetrahydrofolic acid

The finding that the *Streptococcus lactis* R (SLR) (74) and citrovorum factors (61, 8, 40, 50, 54, 11, 12, 58) were formylated compounds structurally related to folic acid (Fig. 4) suggested that folic acid might function as a formyl carrier. The properties of the citrovorum factor, which appears to be 5-formyl-5, 6, 7, 8-tetrahydrofolic acid, suggested that it was an active form of folic acid: unlike the SLR factor, it was generally active in lieu of folic acid but was not inhibited by folic acid inhibitors (70). However, the possibility that its formyl group was the "C<sub>1</sub>" unit involved in



serine biosynthesis appeared doubtful, in view of the evidence presented by Elwyn et al. (23, 65) that the removal of the serine- $\beta$ -carbon did not involve obligatory oxidation to a formyl group. The ratio of  $C^{14}$  to D of the hydroxymethyl group of serine was little changed on its conversion to choline methyl groups. Since serine preparations possessing different orientations of deuterium on the  $\beta$ -carbon were employed in this study, it was not possible to account for the results on a steric basis, by the assumption that oxidation of the serine- $\beta$ -carbon to the formyl level in its conversion to choline methyl groups removed only normal hydrogen. The existence of a non-enzymatic formation of serine from glycine catalyzed by pyridoxal and alum (41, 42) also suggested that the biosynthesis involved a formaldehyde derivative rather than a formyl intermediate. In consideration of the findings of Elwyn, Weissbach, and Sprinson, Welch and Nichol (71) proposed that in addition to a formyl derivative of tetrahydrofolic acid there existed a reduced form, 5-hydroxymethyltetrahydrofolic acid (Fig. 4).

The formulation of serine biosynthesis as a reaction involving a condensation between the  $\alpha$ -carbon of glycine, activated by Schiff base formation with pyridoxal phosphate, and N<sup>5</sup>-hydroxymethyltetrahydrofolic acid was of interest in that this process resembled the well-known Mannich reaction. Serine would be produced by hydrolytic cleavage of the resulting product. This hypothesis has been tested by using phosphate buffer extracts of pigeon liver (34). The cofactor requirements of the serine-biosynthesizing enzyme were studied by incubating the pigeon liver extracts anaerobically with 1- $C^{14}$ -glycine and L-serine. The  $\beta$ -carbon of the L-serine provided the " $C_1$ " unit (reaction 2) for the conversion of 1- $C^{14}$ -glycine to 1- $C^{14}$ -serine (reaction 3). This procedure separated the cofactor



requirements of the serine-biosynthesizing enzyme from those involved in the conversion of formaldehyde and formate to the active  $C_1$  unit.

Pigeon liver extracts were found to incorporate glycine-C<sup>14</sup> into serine. The preparation used in the experiment shown in Table 6

TABLE 6  
INCORPORATION OF GLYCINE-1-C<sup>14</sup> INTO SERINE IN PIGEON LIVER EXTRACT (35)

Preparation	Additions	Activity of Serine (c. p. m.)
Untreated extract	None	200
Untreated extract	THFA	7,600
Dowex-treated, dialyzed extract	THFA	7,600

1 ml. untreated or Dowex-1 (chloride)-treated, dialyzed (15 hrs.) extract. 3.5  $\mu$ M. glycine-1-C<sup>14</sup>, 16,600 c. p. m., 8  $\mu$ M. L-serine, 3.5  $\mu$ M. tetrahydrofolic acid (THFA). 2 ml. total volume.

had been stored for several months and had retained relatively little enzymatic activity. The addition of tetrahydrofolic acid (THFA)<sup>4</sup> produced a marked increase in the incorporation of glycine-C<sup>14</sup> into serine. After treatment of the extract with Dowex-1 (chloride) and dialysis, the addition of THFA restored the activity to that of the untreated extract + THFA.

The additions of ATP, pyridoxal phosphate, or homocysteine did not increase the rate of the THFA-catalyzed reaction. Similar results have recently been reported by Blakely (6).

TABLE 7  
STIMULATION OF THE INTERCONVERSION OF SERINE AND GLYCINE  
IN PIGEON LIVER EXTRACT (35)

Additions	Activity of Serine (c. p. m.)
None	1,180
Homocysteine	1,200
THFA	2,770
THFA (45 min.)	4,130

0.5 ml. Dowex-1 (chloride)-treated, dialyzed (22 hrs.) extract. 10  $\mu$ M. glycine-1-C<sup>14</sup>, 11,000 c. p. m., 10  $\mu$ M. L-serine, 3.5  $\mu$ M. tetrahydrofolic acid (THFA), 10  $\mu$ M. ATP, 5  $\mu$ M. pyridoxal phosphate, 5  $\mu$ M. homocysteine. Total volume 2 ml. 11 min. incubation under H<sub>2</sub> at 34° C.

<sup>4</sup> Prepared by the procedure of O'Dell et al. (48).



The results of these studies in which the interconversion of serine and glycine in Dowex-treated, dialyzed extracts was strongly activated by the sole addition of THFA are consistent with the formulation of the serine biosynthesis reaction as a condensation between hydroxymethyl-THFA and activated glycine, followed by hydrolytic cleavage of the resulting product.<sup>5</sup> The failure of added pyridoxal phosphate to stimulate the reaction does not indicate that it is not involved in this process. In view of the difficulty of resolving pyridoxal phosphate enzymes into apoenzyme and coenzyme, it was not expected that this would be achieved by treatment of the enzyme by Dowex-1 and dialysis.

In the course of studies of sarcosine metabolism in rat liver, studies which have been made of the interconversion of serine and glycine in this preparation are in agreement with the implications of the pigeon liver studies (14). As shown in Table 8, the incorporation

TABLE 8

EFFECT OF TETRAHYDROFOLIC ACID (THFA) ON THE INTERCONVERSION OF SERINE AND GLYCINE IN WASHED RAT LIVER PARTICLES (14)

Additions	Activity of Serine (c. p. m.)			
	15 min.	30 min.	60 min.	90 min.
None	1,900	4,100	7,500	9,550
THFA	2,100	3,500	7,100	10,200

2 ml. particles corresponding to 2 ml. of a 1 : 2 homogenate of rat liver in phosphate buffer; 5  $\mu$ M. glycine-1-C<sup>14</sup>, 40,000 c. p. m., 10  $\mu$ M. L-serine, 1  $\mu$ M. Mn<sup>++</sup>, 3.5  $\mu$ M. THFA. Total volume 2.35 ml. Incubation at 34° C. under H<sub>2</sub>.

of glycine-C<sup>14</sup> into serine by washed particles was not increased by the addition of THFA. On the other hand a desoxycholic-acid extract of the particles incorporated no glycine unless THFA was added

<sup>5</sup> Hydroxymethyl-THFA may also be involved as a cofactor in labile methyl and thymine methyl biosynthesis by processes similar to that proposed for serine formation. The transfer of serine- $\beta$ -carbon to choline and thymine methyl groups without loss of carbon-bound hydrogen (25) suggests that these processes involve a C<sub>1</sub> unit at the oxidation level of formaldehyde. Methionine methyl synthesis may involve a Mannich type reaction between hydroxymethyl-THFA and homocysteine, followed by either reductive cleavage of the N-S bond, or a dissociation in which a dihydrofolic acid is formed. A similar type of process in which cytidine or desoxycytidine replaces homocysteine is suggested in thymine biosynthesis (25).

TABLE 9  
EFFECT OF TETRAHYDROFOLIC ACID (THFA) ON SERINE BIOSYNTHESIS  
IN RAT LIVER PREPARATIONS (14)

Preparation	Addition	Activity of Serine (c. p. m.)
Washed particles	None	13,240
" "	THFA	14,000
Supernatant	None	0
" "	THFA	3,200
Solubilized particles	None	0
" "	THFA	11,500
Boiled " "	THFA	0

2 ml. washed particles corresponding to 2 ml. of a 1 : 2 homogenate of rat liver in phosphate buffer; 5  $\mu$ M. glycine-1-C<sup>14</sup>, 40,000 c. p. m., 10  $\mu$ M. L-serine, 4.9  $\mu$ M. THFA, 2  $\mu$ M. Mn<sup>++</sup>. Total volume 2.57 ml. 45 min. incubation under H<sub>2</sub> at 34° C.

(Table 9). When added at its optimal concentration, THFA stimulated the interconversion reaction in the liver-particle extract at a rate comparable to that of the original particles. These observations indicate that the enzyme of rat liver particles possesses a cofactor that is not readily removed by washing, and that this cofactor is destroyed or that its effective concentration is decreased on solubilization of the enzyme. As observed with the pigeon liver extract, ATP did not affect the rate of incorporation of glycine-C<sup>14</sup> into serine in preparations stimulated by THFA (Table 10). Dihydrofolic acid (DHFA)<sup>4</sup>

TABLE 10  
EFFECT OF ATP ON THE INTERCONVERSION OF SERINE AND GLYCINE  
IN RAT LIVER EXTRACT (14)

Additions	Activity of Serine (c. p. m.)	
	10 min.	20 min.
None	0	0
ATP	0	0
THFA	1,350	2,250
ATP + THFA	1,310	2,100

ml. extract, 5  $\mu$ M. glycine-1-C<sup>14</sup>, 25,000 c. p. m., 10  $\mu$ M. L-serine, 1  $\mu$ M. Mn<sup>++</sup>, 5  $\mu$ M. THFA, 10  $\mu$ M. ATP. Total volume 2.45 ml. Incubation under H<sub>2</sub> at 34° C.



also reactivated the incorporation of glycine- $C^{14}$  into serine but the maximal stimulation of THFA was nearly twice that of DHFA and occurred at lower concentration.

The incorporation of formaldehyde into serine- $\beta$ -carbon was subsequently studied by incubating pigeon liver extracts with  $C^{14}$ -formaldehyde and glycine and determining the activity incorporated into serine (34).

Formaldehyde- $C^{14}$  utilization in the Dowex-treated and dialyzed pigeon liver extract was stimulated by the sole addition of THFA (Table 11). Colorimetric determination of serine by the procedure

TABLE 11

INCORPORATION OF FORMALDEHYDE- $C^{14}$  INTO SERINE IN PIGEON LIVER EXTRACT (35)

Preparation	Additions	Activity of Serine (c. p. m.)
Dowex treated-dialyzed	None	180
" " "	THFA	112,000
" " "	DHFA	7,500
Untreated	None	1,350
"	THFA	96,000
"	DHFA	47,000

1 ml. extract treated twice with Dowex-1 (chloride) and dialyzed (24 hrs.).  $6 \mu M$ .  $C^{14}$ -HCHO, 280,000 c. p. m.,  $10 \mu M$ . glycine,  $10 \mu M$ . ATP,  $5 \mu M$ . homocysteine,  $3.5 \mu M$ . tetrahydrofolic acid (THFA),  $3.5 \mu M$ . dihydrofolic acid (DHFA). Total volume 2 ml. 13 min. incubation at  $34^\circ C$ .

of Frisell et al. (27) indicated the occurrence of a corresponding increased net formation of serine. The incorporation of formaldehyde- $C^{14}$  into serine under these conditions was similar to that produced by untreated pigeon liver extracts stimulated by THFA.

ATP did not increase the rate of incorporation of formaldehyde- $C^{14}$  into serine, and homocysteine was actually inhibitory. The latter effect may be accounted for by a non-enzymatic reaction of formaldehyde and homocysteine (3).

As shown in Table 11, dihydrofolic acid (DHFA) also stimulated the incorporation of formaldehyde- $C^{14}$  in a Dowex-treated-dialyzed pigeon liver extract, but the effect was much less pronounced than

that of THFA. The effect of DHFA was similar to that of THFA when ATP, DPN,  $Mn^{++}$ , and glucose-6-phosphate (G-6-P) were added. These substances were considered to generate DPNH for the reduction of DHFA to THFA by reconstituting the glycolytic system in this preparation. As would be expected, the stimulatory effect of DHFA was much more pronounced in untreated than in treated extracts.

The addition of THFA also stimulated the incorporation of formaldehyde- $C^{14}$  into serine in washed rat liver particles and in desoxycholic-acid extracts of the particles. These preparations possessed little or no ability to utilize formaldehyde for serine- $\beta$ -carbon formation but showed a definite utilization upon the addition of THFA (Table 12). Seemingly conflicting evidence has been reported con-

TABLE 12  
INCORPORATION OF FORMATE- $C^{14}$  INTO SERINE IN PIGEON LIVER EXTRACT (35)

Preparation	Additions	Activity of Serine (c. p. m.)
Dowex treated-dialyzed	None	20
" " "	homocysteine	290
" " "	THFA	220
" " "	THFA, ATP, DPN, $Mn^{++}$ + G-6-P	79,000
" " "	ATP, DPN, $Mn^{++}$ + G-6-P	180
Untreated	None	1,800
"	homocysteine	7,400
"	THFA	79,000
"	THFA, ATP, DPN, $Mn^{++}$ + G-6-P	86,000

1.0 ml. untreated or Dowex-1 (chloride) treated, dialyzed (24 hrs.) extract.  $5 \mu M$ .  $C^{14}$ -formate, 268,000 c. p. m.,  $10 \mu M$ . ATP,  $2 \mu M$ . DPN,  $20 \mu M$ . G-6-P,  $2 \mu M$ .  $Mn^{++}$ ,  $5 \mu M$ . DL-homocysteine,  $5 \mu M$ . THFA. Total volume 2 ml. 30 min. incubation under  $H_2$  at  $34^\circ C$ .

cerning the ability of mitochondria to incorporate formaldehyde- $C^{14}$  into serine. The rat liver particles studied by Mitoma and Greenberg, consisting of "virtually uncontaminated mitochondria" (44), incorporated appreciable formaldehyde- $C^{14}$  into serine. However, Mackenzie et al. (39) have reported that rat liver mitochondria are unable to carry out this process. The apparent discrepancy



between the findings of Mitoma and Greenberg and those of Mackenzie et al. may be due to differences in methods of handling the liver preparations resulting in different degrees of loss or destruction of cofactor. The observation of Mitoma and Greenberg (44) that ATP plus citrate stimulated the incorporation of formaldehyde- $C^{14}$  into serine under anaerobic conditions may have been due to reduction of an oxidized precursor of the cofactor of serine biosynthesis by the limited operation of the tricarboxylic acid cycle.

The difference in the effect of THFA on the incorporation of formaldehyde- $C^{14}$  into serine in washed liver particles, a process which is stimulated, and on the interconversion of glycine and serine, a process which is not significantly affected, may be accounted for by a hypothesis in which the interconversion enzyme is assumed to be closely associated with sarcosine oxidase and to utilize both a bound and a dissociable cofactor. According to this interpretation, the bound coenzyme is principally involved in the conversion of sarcosine to serine, which is an efficient process in mitochondria (44). Sarcosine provides both a " $C_1$ " unit (from its methyl group) and glycine for serine formation (44,39). Formaldehyde utilization is assumed here to require a dissociable carrier. The addition of THFA in the usual concentrations would not materially increase the effective coenzyme concentration of the interconversion enzyme and would not stimulate the process. However, such addition would be required for the formation of active  $C_1$  units from formaldehyde in preparations depleted of cofactor by repeated washing.

The mechanism of the utilization of formate- $C^{14}$  for serine- $\beta$ -carbon formation has been investigated by the procedure employed in the formaldehyde studies. Berg (4) found that phosphate buffer pigeon liver extracts rapidly incorporate formate- $C^{14}$  into serine- $\beta$ -carbon. This conversion was strongly stimulated by homocysteine and abolished by Dowex-1 treatment. It was not expected that the single addition of THFA would reactivate Dowex-treated-dialyzed extracts toward the incorporation of formate carbon into serine, since this process involved the reduction of a single-carbon unit from the oxidation level of formate to that of formaldehyde. In accord-

ance with this expectation, the single addition of THFA was found to produce relatively little effect. However, the addition of THFA together with ATP, DPN, G-6-P, and  $Mn^{++}$  strongly stimulated the fixation of formate carbon into serine. Colorimetric determination of serine by the procedure of Frisell et al. (27) indicated the occurrence of a corresponding increased net formation of serine.  $C^{14}$  incorporated into serine, under these conditions, was greater than that of the untreated extracts stimulated by homocysteine and was comparable to that which was obtained when THFA, ATP, DPN, G-6-P, and  $Mn^{++}$  were added to untreated pigeon liver extracts. Formate- $C^{14}$  was not incorporated into any non-volatile substance in treated

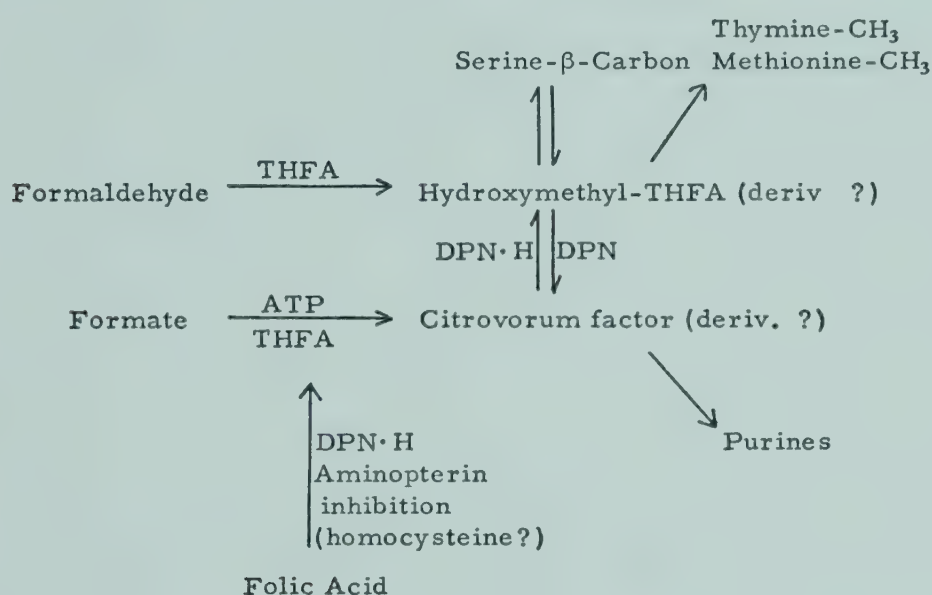


FIG. 5. Proposed Mechanism of Formate and Formaldehyde Utilization in Serine Formation.

extracts in the absence of ATP or of THFA. When G-6-P alone was omitted, formate- $C^{14}$  was rapidly incorporated into some substance or substances of unknown identity. When DPN or  $Mn^{++}$  was omitted, the incorporation of formate- $C^{14}$  into serine was reduced but not completely abolished.

Folic acid stimulated the incorporation of formate- $C^{14}$  into serine when added with ATP, DPN, G-6-P, and  $Mn^{++}$ , but the serine activity was considerably less than that obtained with THFA (35).

The incorporation of formate- $C^{14}$  into serine- $\beta$ -carbon has been tentatively postulated to occur via the mechanism shown in Fig. 5. In this scheme formate combines with THFA in the presence of



ATP to form citrovorum factor, which is subsequently reduced to 5-hydroxymethyltetrahydrofolic acid by a DPN enzyme system. DPN is also considered to function as the cofactor of the conversion of folic acid to THFA.

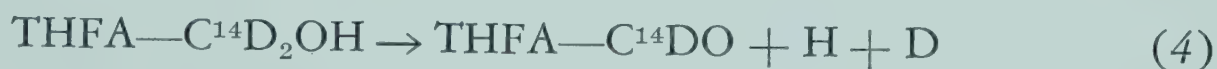
The observed effects of the single omission of THFA, ATP, DPN, or G-6-P on the fixation of formate- $C^{14}$  and its incorporation into serine- $\beta$ -carbon in treated pigeon liver extract are consistent with this scheme. The lack of formate- $C^{14}$  fixation in the absence of ATP or THFA are in accord with the involvement of these substances in the initial phase of formate utilization. The omission of added DPN or G-6-P would not be expected to prevent the fixation of formate carbon, but the incorporation of  $C^{14}$  into serine would be reduced to a degree depending on the availability in the extract of DPN and of substrates for DPN reduction.

The proposed scheme is consistent with available reports concerning the metabolic relationships of compounds containing a single-carbon unit. It is in agreement with the indication that formate and formaldehyde do not undergo obligatory interconversion in their incorporation into methionine methyl groups (4) or purine carbons 2 and 8 (29), but are metabolized via a common intermediate. It is also in accord with the findings of Nichol (46) that ATP, DPN, and  $Mg^{++}$  are required for formation of citrovorum factor in cell-free extracts of *S. faecalis* in the presence of ascorbate, formate, and glucose.

The metabolism of single carbon compounds by a pathway via citrovorum factor or hydroxymethyl-THFA (or both), which undergo interconversion, may appear to be inconsistent with tracer studies in which the carbon of deuterio- $\beta$ -,  $C^{14}$ -labeled serine and deuterio,  $C^{14}$ -formate was incorporated into choline (51, 23, 65, 25) and thymine methyl groups (23, 65) with little loss of deuterium. However, this evidence may be reconciled with the proposed scheme of  $C_1$  metabolism. In the case of the formate utilization, the interconversion of citrovorum factor and hydroxymethyl-THFA would not be expected to labilize the C-bound deuterium. It would be anticipated that hydrogen would be added to the formyl group in the reduction



of citrovorum factor with a single spatial distribution and that the same hydrogen atom would be removed in the reversal of this process. In the case of serine, the reports that serine- $\beta$ -carbon was transformed to choline and thymine methyl groups with little loss of hydrogen provides strong evidence that a formyl intermediate is not involved in these processes. The results are not, however, inconsistent with the existence of an hydroxymethyltetrahydrofolic acid (THFA-CH<sub>2</sub>OH) intermediate which can undergo reversible conversion to citrovorum factor (THFA-CHO). Ratios of C<sup>14</sup>:D dilution for the conversion of serine- $\beta$ -C<sup>14</sup>D<sub>2</sub>OH to choline methyl groups, 0.97 and 0.89 (23), and to thymine methyl groups, 0.75 (23) 0.78 (65), are consistent with the occurrence of some interconversion of THFA-CH<sub>2</sub>OH and THFA-CHO. These values are to be compared with the ratio of 0.50 which would be expected for maximum loss of deuterium by this process. The value of 0.50 has been obtained by the following reasoning. Half of the deuterium of THFA-C<sup>14</sup>D<sub>2</sub>OH would be removed by the conversions:



Repetition of this process would not result in further loss of deuterium, since the hydrogen atom with a single spatial distribution would be removed and added.

It should be noted that THFA-C<sup>14</sup>DO may be highly diluted by a pool of unlabeled citrovorum factor. The THFA-C<sup>14</sup>DHOH produced from it may contribute relatively little C<sup>14</sup> or D to methyl groups compared with the THFA-C<sup>14</sup>D<sub>2</sub>OH directly formed from serine. Under these conditions THFA-CH<sub>2</sub>OH and THFA-CHO could undergo extensive interconversion with little effect on the ratio of C<sup>14</sup>:D dilution of the thymine and choline methyl groups.

Aminopterin appears to inhibit the metabolism of single carbon compounds by interfering with the reduction of folic acid to THFA. Blakely (6) has recently reported that this substance inhibited the interconversion of serine and glycine in pigeon liver extract stimulated by folic acid, but not by THFA. This finding is consistent



with the prior observation of Nichol and Welch that aminopterin reduced the conversion of folic acid to citrovorum factor in liver (47). These investigators have also reported that ascorbic acid promotes the latter reaction (47).

The place of homocysteine in this scheme is problematical. Berg (4) observed that homocysteine and homocystine strongly stimulated the incorporation of formate- $C^{14}$  into serine- $\beta$ -carbon in untreated pigeon liver extracts. This stimulation was of a higher order of magnitude than that produced by other reducing agents, and it was therefore tentatively postulated that the conversion of formate to serine- $\beta$ -carbon involves S-formyl and S-hydroxymethyl-homocysteine as intermediates. Deodhar subsequently found that homocysteine stimulates and cysteine inhibits the incorporation of formate- $C^{14}$  into serine- $\beta$ -carbon in untreated chicken liver extracts (14). Homocysteine does not, however, stimulate the interconversion of serine and glycine or the utilization of formaldehyde and formate in Dowex-treated, dialyzed extracts when these reactions are stimulated by THFA (35). It is possible that homocysteine is involved in the reduction of folic acid to tetrahydrofolic acid. Doctor et al (17) have reported that homocysteine increased the conversion of folic acid to citrovorum factor, but that glutathione, ascorbic acid, and cysteine were without effect.

There appears to be a substantial possibility that the physiological  $C_1$ -carrier is a bound form of THFA rather than the free substance. Folic acid and citrovorum factor both occur largely in bound form (15, 32, 72, 16, 9). The folic acid derivatives that have been identified are polyglutamates, and there is some indication that the citrovorum factor derivatives possess similar structures. The further possibility that THFA is substituted at position 8 is of interest in that this might protect the reduced pteridine ring toward oxidation like the 5-formyl group in citrovorum factor. However, there is no evidence as to the existence of such a compound, and it is possible that nuclear oxidation and reduction are involved in some processes in which folic acid derivatives are concerned.

SIGNIFICANCE OF THE SERINE-GLYCINE INTERCONVERSION  
REACTION

The interconversion of glycine and serine is a rapid process in the rat. It has been a repeated observation that after the administration of labeled glycine or serine, the isotope concentration of corresponding parts of these two amino acids in the liver are similar (Table 13). Thus, Goldworthy et al. (28) found that the specific

TABLE 13

LABELING OF GLYCINE AND SERINE IN THE RAT PRODUCED BY THE  
LABELED AMINO ACIDS

Amino Acid Administered	Time <sup>1</sup> (hrs.)	Labeling of the Amino Acid Isolated				Reference
			N	1-C	2-C	
1-C <sup>14</sup> -glycine	21	serine	—	4,400 <sup>2,4</sup>	—	Goldworthy et al. (28) <sup>4</sup>
		glycine	—	3,800 <sup>2,4</sup>	—	
N <sup>15</sup> ,1-C <sup>13</sup> -glycine	14	serine	2.32 <sup>3</sup>	0.78 <sup>3</sup>	—	Sakami (56)
		glycine	2.45 <sup>3</sup>	0.78 <sup>3</sup>	—	
2-C <sup>14</sup> -glycine	14	serine	—	—	365 <sup>2</sup>	Sakami (56)
		glycine	—	—	375 <sup>3</sup>	
N <sup>15</sup> -serine	2	serine	5.23 <sup>3</sup>	—	—	Aqvist (1)
		glycine	3.98 <sup>3</sup>	—	—	
N <sup>15</sup> -serine unspecified		serine	3.98 <sup>3</sup>	—	—	Arnstein and Neuberger (2)
		glycine	4.53 <sup>3</sup>	—	—	

<sup>1</sup> Time of sacrifice of animals after last administration of labeled compound.

<sup>2</sup> c. p. m. per mg. of labeled carbon.

<sup>3</sup> Atoms per cent excess.

<sup>4</sup> Calculated from the data of Goldworthy et al.

activities of liver glycine and serine determined 21 hours after the administration of 1-C<sup>14</sup>-glycine were approximately equal. In studies of the glycogenic action of glycine with nitrogen- and carbon-labeled glycines, the corresponding positions of the two amino acids were found to be equally labeled within 14 hours after the feeding of the labeled compounds (56). Similar results have been obtained with labeled serine. When N<sup>15</sup>-serine has been administered high labeling of the liver glycine has been observed (1, 2). Other similar observations have been made by Elliott and Neuberger (20), Aqvist (1),



Elwyn and Sprinson (24), and Arnstein and Neuberger (2). The rapidity of the interconversion of glycine and serine in rats is also indicated by the efficiency with which serine- $\beta$ -C<sup>14</sup> is utilized for the formation of guanine carbons 2 and 8, and the thymine methyl carbon (22).

The interconversion of glycine and serine in the pigeon liver likewise appears to be a rapid process, as indicated by the efficiency with which the 3-C<sup>14</sup> and N<sup>15</sup> of labeled serine were incorporated into the positions of the uric acid specifically labeled by formate and glycine (21).

Since the incorporation of isotope from one compound into another may occur by exchange processes, these data from tracer studies do not indicate *per se* the occurrence of a net conversion of glycine to serine or of serine to glycine under the conditions of the experiments. Thus, glycine-N<sup>15</sup> could be efficiently incorporated into serine by transfer of a C<sub>1</sub> unit from unlabeled serine to N<sup>15</sup>-glycine, a process involving no net formation of serine. Similarly, exchange of labeled serine- $\beta$ -carbon with the C<sub>1</sub> pool would label substances formed from one carbon units without obligatory net conversion of serine to glycine. The extensive introduction of glycine N<sup>15</sup> into serine and the high labeling of the purine 2 and 8 positions produced by serine- $\beta$ -carbon merely suggests that under certain conditions the corresponding net conversions may occur. There is at present no completely conclusive evidence as to whether the interconversion of serine and glycine operates under normal conditions to effect a net reduction in C<sub>1</sub> units and glycine, and a net formation of serine, or in the reverse manner.

Apart from any significance that the interconversion reaction may have in the overall supply of glycine and C<sub>1</sub> units or of serine to the organism, there is the possibility that it possesses a ministrative function in the collection, storage, and distribution of single carbon units. For example, serine formation may trap C<sub>1</sub> units formed in sarcosine oxidation by liver mitochondria for purine and thymine formation in peripheral tissues, or until the slow direct oxidative processes of liver can accommodate them. This type of function

would provide a plausible explanation for the widespread distribution of the interconversion reaction. Homogenates of all animal tissues that have been examined, liver, kidney, spleen, adrenals, pituitary, thyroid, thymus, pancreas, testes, skeletal muscle, heart, and intestine have been found to incorporate glycine-C<sup>14</sup> into serine after the addition of tetrahydrofolic acid (14).

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# CONVERSION OF N-METHYL GLYCINES TO ACTIVE FORMALDEHYDE AND SERINE \*

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THE METABOLIC origin of one-carbon compounds and their participation in synthetic reactions is a new field in biochemistry. Until several years ago there was meager evidence, and little or no speculation, concerning the production of formaldehyde and formate by higher organisms. Thus, while the occurrence of small amounts of formic acid in the urine of man and other animals had been frequently reported, information concerning its urinary precursors and its production within the body was very scanty, as is shown in the experiments of Dakin and his colleagues (1) on formate excretion. With respect to the occurrence of formaldehyde in animals, the evidence was even more indirect and remote. Abbott and H. B. Lewis (2) and Bloch and Schoenheimer (3) described the demethylation of sarcosine to glycine in the intact organism 15 years ago, and a year later Handler, Bernheim, and Klein (4) reported a positive color test for formaldehyde when sarcosine was incubated with a liver sediment. However, there was no evidence at that time that sarcosine itself is a normal constituent of the organism. Moreover, these investigations were not pursued with respect to a more specific

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identification of the product of its demethylation, and Handler and coworkers (4) raised the question themselves as to whether formaldehyde was formed from sarcosine *in vivo*. Actually, formate and formaldehyde had received most attention as products of methyl alcohol metabolism, with the acute toxicity of methanol being ascribed to the formation of one or the other of these compounds. Even in this area there was some question concerning formaldehyde formation, and usually the production of formate by an unspecified pathway was held responsible for the symptoms of methanol poisoning (5).

In 1947, when the extensive oxidation of biologically labile methyl groups to carbon dioxide in the intact animal was first demonstrated, we called attention to the metabolic importance of the intermediates formed in this reaction (6, 7), for it seemed probable to us that one pathway of methyl catabolism would involve formaldehyde and formate, though transfer of the methyl carbon to the carbon chain of another compound, prior to oxidation, was by no means excluded. In 1949 du Vigneaud and I reported the isolation of radio formaldehyde and the identification of radio formate as products of the oxidation of sarcosine labeled in the methyl group with  $C^{14}$ , and pointed out that these findings opened a new field for exploration and study, namely the origin and metabolism of one-carbon compounds (8). It was further pointed out at that time that the quantitative aspects of sarcosine metabolism suggested the formation of an active "one-carbon fragment" paralleling the then popular "two-carbon fragment" in its participation in synthetic reactions.

Subsequent studies have borne out the latter hypothesis. They have also indicated that in keeping with the versatility of living systems methyl groups may be converted to carbon dioxide by a circuitous route involving the carbon chains of serine, choline, and betaine, as well as by the more direct route involving formaldehyde and formate. In all of these reactions the N-methyl glycines, dimethylglycine and sarcosine, occupy key positions. Furthermore, the delineation of their interrelationship has completed a cyclic series of reactions whereby methyl groups are metabolized and one-carbon



compounds, possessing different synthetic potentials, are generated. In describing the results of our experiments in this area, no attempt will be made to review in detail the fundamental contributions to the fields of glycine, serine, or one-carbon metabolism of the other investigators who are members of this symposium.

### DEMONSTRATION OF $\text{CH}_2\text{O}$ AND $\text{HCOOH}$ PRODUCTION IN THE ANIMAL ORGANISM

Following the demonstration of the oxidation of labile methyl groups to  $\text{CO}_2$  in the intact animal (6, 7), it was found (Table 1)

TABLE 1  
OXIDATION OF METHYL GROUP BY RAT LIVER AND KIDNEY CORTEX SLICES

3.35 $\mu\text{M}$ . radioactive substrate per 200 mg. tissue slices in Krebs-Ringer-phosphate, pH 7.4.				
Tissue	% $-\text{C}^*\text{H}_3$ oxidized to $\text{C}^*\text{O}_2$ per hour			
	Methionine	Choline †	Betaine †	Sarcosine
Liver	3.48	0.45	0.38	5.56
Kidney cortex	1.37	<0.02	<0.02	6.90

† One methyl group labelled per molecule.

that methionine, choline, betaine, and sarcosine labeled with  $\text{C}^{14}$  in their methyl groups are converted to  $\text{C}^{14}\text{O}_2$  by surviving liver slices (9, 10). However, when the cells were broken in a Potter-Elvehjem homogenizer, only the methyl group of sarcosine was oxidized. Since cell-free preparations offer many advantages in the isolation of reaction products, sarcosine was employed as the source of methyl groups in the subsequent isolation experiments. In concomitant experiments it was shown that sarcosine is a normal metabolite.

*Metabolic formation of sarcosine from labile methyl groups.* Methylglycine was first prepared in 1847 by Liebig (11) as a product of creatine degradation by a concentrated solution of barium hydroxide. Liebig named the new compound sarcosine because of its chemical derivation from creatine, and not, as has often been inferred, because he ever obtained it from muscle. While many

experiments on the administration of sarcosine and the nature of its excretory products had been conducted since Liebig's description of the compound, we were unable to find any reference to its occurrence in nature except the report of Kossel and Edlbacher (12) on the isolation of sarcosine, together with several other amino acids, from the semiautolyzed radial caeca of the starfish. Since 100 years had passed since its discovery and sarcosine still had not been identified as a metabolite, it was highly desirable that evidence be obtained for its formation in higher animals if it were to be used as a prototype of methyl groups in the isolation experiments. This problem was approached in a collaboration with Horner (13) by applying the isotope carrier technique to the intact animal. Rats were fed sarcosine at a level that insured its excretion in the urine. At the same time  $C^{14}H_3$ -methionine or betaine was administered orally or parenterally, and the sarcosine excreted in the urine during the next 24 hours was isolated as the  $\beta$ -naphthalene-sulfonyl derivative. This compound proved to be highly radioactive, and as shown in Table 2,

TABLE 2

SPECIFIC ACTIVITY OF SARCOSINE ISOLATED AS  $\beta$ -NAPHTHALENESULFONYL DERIVATIVE FROM URINE OF RATS FED BETAINE OR METHIONINE LABELED WITH  $C^{14}$  IN METHYL GROUP

Sarcosine derivative	Compound fed	
	Radiobetaine	Radiomethionine
	<i>c.p.m. per mM.</i>	<i>c.p.m. per mM.</i>
$\beta$ -Naphthalenesulfonylsarcosine .....	$3.2 \times 10^4$	$5.8 \times 10^3$
Sarcosine hydrochloride .....	$3.2 \times 10^4$	$5.3 \times 10^3$
Creatine hydrate .....	$3.4 \times 10^4$	$5.2 \times 10^3$
Creatinine chloride .....	$3.2 \times 10^4$	$5.4 \times 10^3$
"    potassium picrate .....	$3.2 \times 10^4$	$5.8 \times 10^3$
Methylamine chloroplatinate .....	$3.5 \times 10^4$	$6.3 \times 10^3$

a series of derivatives prepared from it possessed a constant specific activity. These results indicated that sarcosine is a metabolite in the rat and that biologically labile methyl groups as a class contribute to its formation. Consequently sarcosine could be employed to study the intermediates formed in methyl metabolism.



*Radioactive formaldehyde and formate from radio sarcosine.* In concomitant experiments with those described above I was able to isolate radioformaldehyde as the dimedon derivative (Table 3) when

TABLE 3

OXIDATION OF  $-\text{C}^{14}\text{H}_3$  SARCOSINE TO  $\text{HC}^{14}\text{HO}$ ,  $\text{HC}^{14}\text{OOH}$  AND  $\text{C}^{14}\text{O}_2$

	Time Hrs.	Products as % added $-\text{C}^{14}\text{H}_3$		
		$\text{C}^{14}\text{H}_2\text{O}$	$\text{HC}^{14}\text{OOH}$	$\text{C}^{14}\text{O}_2$
Liver Particles	1	17	10	1
Liver Slices	2	1	2	5
Intact Rat	24	(Trace)	0.3	50

radiosarcosine was incubated with partially sedimented liver homogenates or with liver slices (10). Formaldehyde was also isolated from homogenates without the use of a carrier, to remove any doubts concerning its identity. Because of the manifold implications of the role of such a reactive molecule as formaldehyde in synthetic reactions, precautions were taken to obviate the possibility that it was formed from a labile precursor during the isolation procedure. In experiments employing both homogenates and liver slices, formaldehyde was blown out of the incubation mixture with a stream of nitrogen, thus furnishing convincing evidence that at least part of the formaldehyde formed from the methyl group of sarcosine exists in a free form.

Radioformate was also identified as a product of methyl metabolism in both liver homogenates and liver slices. Evidence that the incubation mixtures contained free formate was obtained by making the contents of the Warburg flasks slightly acid with  $\text{H}_3\text{PO}_4$  and distilling under reduced pressure at  $22^\circ\text{C}$ . The yield of radioformic acid was comparable to that obtained under the more drastic conditions usually employed.

These observations in vitro were then extended to the intact animal (10). Following the administration of radiosarcosine to rats,  $\text{HC}^{14}\text{OOH}$  and traces of  $\text{C}^{14}\text{H}_2\text{O}$  were eliminated in the urine. Also, the conversion of the tagged methyl group of injected sarcosine to  $\text{C}^{14}\text{O}_2$  was found to be most extensive and rapid, the peak of the

oxidation occurring within the hour, with 40 per cent of the injected  $C^{14}$  being eliminated in 6 hours. These experiments, which are summarized in Table 3, indicate that formaldehyde and free formate are naturally occurring compounds in higher animals. Moreover, they demonstrate that biologically labile methyl groups as a class are sources of formaldehyde and formate in the animal organism. On the basis of these observations, the oxidation of the methyl group of sarcosine, and of methyl groups contributing to it, was formulated (10) as follows:



where  $X$  represents the initial product(s) formed in the reaction. In presenting these consecutive reactions it was emphasized that such a reactive molecule as formaldehyde would not be converted entirely to formate, but that a considerable part would be "found to play an important role in synthetic reactions, particularly in reactions involving proteins and amino acids."

*Synthetic reactions of sarcosine.* While the above formulation provided a pathway for the direct oxidation of methyl groups to carbon dioxide, our experiments also furnished evidence that other and more extensive routes for the metabolism of methyl carbons existed. Although the radiosarcosine incubated with partially sedimented liver preparations disappeared completely and the oxygen uptake was the value calculated for the complete oxidation of the methyl group to formaldehyde (with allowance for the observed production of formate and carbon dioxide), only 25 per cent of the incubated  $C^{14}$  was recovered as  $C^{14}H_2O$ ,  $HC^{14}OOH$ , and  $C^{14}O_2$  (10). Our isolation and analytical procedures for these compounds were essentially quantitative. Sedimenting the homogenate more thoroughly reshuffled the distribution but did not change the sum total of these three 1-C entities; thus, the accumulation of  $HCOOH$  and  $CO_2$  was decreased, while the accumulation of  $CH_2O$  was increased by the same increment (Table 4). The remainder of the incubated  $C^{14}$  (75%) was found to be present in the TCA-soluble fraction of the incubation mixture as a nonvolatile compound(s).



TABLE 4

OXIDATION OF METHYL GROUP OF RADIOSARCOSINE BY LIVER HOMOGENATES

Experiment No.	Radio-sarcosine	Volume removed from centrifuged homogenate	Oxygen consumption		Oxidation products, as per incubated $-C^{14}H_3$		
			Homogenate alone	Increase due to radiosarcosine	$C^{14}H_2O$	$HC^{14}OOH$	$C^{14}O_2$
	$\mu M.$	per cent	$\mu M.$	$\mu M.$			
41	4.77	20	7.2	3.0	2.4	15.1	8.6
75	3.36	30	6.8	2.1	3.8	14.0	4.6
71A	5.07	20	6.5	2.7	8.1	12.7	2.6
11	5.18	60	2.7	2.8	12.9		1.4
71B	5.07	50 †	2.5	2.4	17.4	10.3	0.5
Controls	4.77–5.7	20	0	0	0	0	0

The addition and reisolation of nonisotopic sarcosine demonstrated that this  $C^{14}$  was not present as radioactive sarcosine. These results, as well as the production of formate, were at variance with the interpretations of Handler and coworkers (4), who, on the basis of oxygen uptake, the increase in amino N as measured by Van Slyke's nitrous acid method, and a color test for formaldehyde, concluded that sarcosine was converted quantitatively to formaldehyde and glycine in washed preparations of ground liver.

The presence of a large amount of a radioactive compound(s) in the TCA-soluble fraction in our experiments suggested that formaldehyde and formate had participated extensively in synthetic reactions. We had already found, in the intact rat, that the methyl carbon of methionine contributed to the synthesis of the adenine of nucleic acid and the heme of hemoglobin (10). Moreover, Sakami, who had previously demonstrated the participation of exogenous formate in the conversion of glycine to serine in the rat (14), showed that the methyl groups of choline could replace formate as the source of the  $\beta$ -carbon of serine (15), a finding consistent with our formulation of the pathway of methyl metabolism.

There was, however, an alternative explanation for the origin of the unknown compound which accounted for 75 per cent of the

incubated  $C^{14}$  in our enzyme system, namely, that it was derived not from formaldehyde or formate, but directly from the precursor of formaldehyde indicated by X in reaction sequence 1. The observed oxygen uptake indicated that such a compound would possess the oxidation level of formaldehyde (10), and the possibility of such a compound was expressed in the hypothesis, cited by du Vigneaud (16), that an active one-carbon fragment, paralleling the active two-carbon fragment, was formed in the oxidation of methyl groups.

In conjunction with the evidence that sarcosine methyl participated extensively in synthetic reactions, it was also found to be a potent source of urinary formate (10). As shown in Table 5, sarcosine was

TABLE 5

EXCRETION OF FORMIC ACID BY RATS FOLLOWING INTRAPERITONEAL INJECTION OF SARCOSINE, GLYCINE, AND METHANOL

Weight of rat	Compound injected	Quantity injected daily	Formic acid excreted in urine during injection period	
			1st day	2nd day
<i>g.</i>		<i>g.</i>	<i>mg.</i>	<i>mg.</i>
237	Sarcosine	1	17.	24
223	"	1	6	
248	"	1	9	13
218	Methanol	0.8	2	7
234	"	0.8	5	24
190	Glycine	1	0.4	0
159	"	1	0.1	1.3

just as effective in this regard as twice its molar level of methanol. Under the same conditions the administration of glycine produced no increase in urinary formate. This was of particular interest, since Siekevitz and Greenberg (17) had reported that the  $\alpha$ -carbon of glycine was oxidized to formate by liver slices, and Siekevitz, Winnick, and Greenberg (18) and Sakami (19) had shown that formate could be replaced by the glycine  $\alpha$ -carbon as a source of the  $\beta$ -carbon of serine. Judging from my experiments with Lubschez, however, the  $\alpha$ -carbon of glycine does not compare in potency with the methyl carbon of sarcosine as a source of formate in the intact



organism (10). Weinhouse and Friedmann (20) have recently confirmed this observation employing isotopic glycine.

The results of the experiments described in this section, and carried out in du Vigneaud's laboratory at Cornell University, indicated that methyl groups were an excellent source of formate and of formaldehyde. The synthetic potentialities of the latter compound appeared to be very great, notwithstanding the exclusive role then assigned by other investigators to formate or a "formate-like" substance. Evidence had been obtained that a compound possessing the oxidation level of formaldehyde participated in synthetic reactions in liver sediments to an extraordinary degree. Finally, formaldehyde and formate offered an attractive pathway for the metabolism of biologically labile methyl groups via sarcosine.

#### SOURCES OF FORMALDEHYDE AND LOCALIZATION OF ENZYMES

When our studies on the origin and metabolism of one-carbon compounds were resumed at the University of Colorado, it was found that formaldehyde accumulates and may be isolated directly as the dimedon derivative, when sarcosine is incubated with an *unfractionated* liver homogenate. In the face of this observation, a number of other compounds were examined, in collaboration with Johnston and Frisell (21), in a similar system prepared in a potassium phosphate buffer designed to resemble the intracellular fluid of liver. Formaldehyde was isolated from four compounds: methanol, dimethylaminoethanol, dimethylglycine, and sarcosine. In earlier experiments Handler and coworkers (4) had obtained a color test for formaldehyde when sarcosine and dimethylglycine were incubated with a liver sediment.

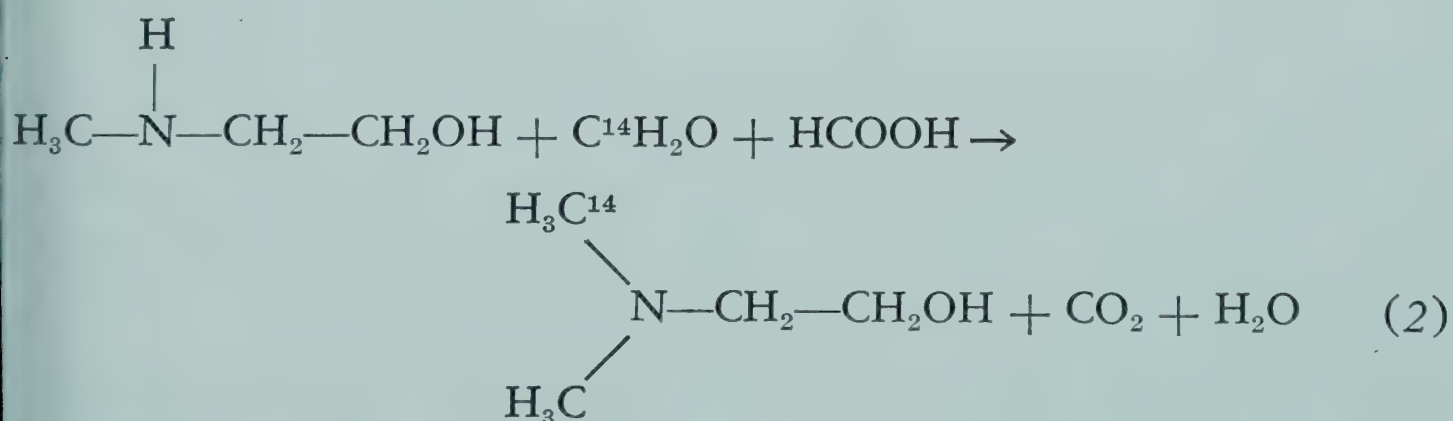
Formaldehyde did not accumulate in our system when the following compounds were incubated with the intact homogenate: aminoethanol, monomethylaminoethanol, choline, betaine, glycine, serine, methionine, and mono- and dimethylamine. These results narrowed considerably the pathway of metabolism of methyl groups in homogenized liver, for it was clear that these compounds could not be major intermediates in the conversion of the former to formaldehyde in this system.

TABLE 6  
DISTRIBUTION OF FORMALDEHYDE-PRODUCING SYSTEMS

Substrate	Mg. Formaldemethone		
	Sup.	Sed.	Recon.
Dimethylaminoethanol	0	0	1
Dimethylglycine	0	6	2
Sarcosine	0	12	6
Methanol	2	0	2

Fractionation of the liver homogenate disclosed (Table 6) that dimethylaminoethanol required both the supernatant and sediment fractions for its conversion to formaldehyde. On the other hand, formaldehyde was isolated when methanol was incubated with the supernatant fraction alone, or when dimethylglycine and sarcosine were incubated with the thoroughly washed sediment.

*Dimethylaminoethanol.* The observation that dimethylaminoethanol was the only one of the N-methylaminoethanols to yield formaldehyde when incubated with liver homogenate prompted Johnston and myself to prepare the radioactive methyl labeled compound. Radiodimethylaminoethanol was synthesized by the method of Clarke and coworkers (22) from monomethylaminoethanol, formate, and radioformaldehyde.<sup>1</sup> The product possessed the same specific activity as the starting formaldehyde. It appears therefore that the role of formate is restricted to that of a reducing agent, and we can formulate the mechanism of this classical methylation reaction as follows:



<sup>1</sup> This and all other isotopic compounds employed in these investigations were obtained on allocation from the Atomic Energy Commission.



It has previously been shown by du Vigneaud and coworkers (23) that the D of deuterioformate ( $\text{DCOOH}$ ) enters the methyl group in such a synthesis.

When the radioactive dimethylaminoethanol was incubated with a whole liver homogenate, radioformaldehyde equivalent to 1.6 per cent of the added  $\text{C}^{14}$  was isolated from the reaction mixture as the dimedon derivative (24). This was equivalent to 3.2 per cent of the incubated methyl groups. Sixty-eight per cent of the original radiodimethylaminoethanol was recovered at the end of the experiment as the picrolonate (Table 7). The oxygen consumption was

TABLE 7

OXIDATION OF  $-\text{C}^{14}\text{H}_3$  DIMETHYLAMINOETHANOL

22.5  $\mu\text{M}$ . of substrate incubated for 2 hours at  $37^\circ\text{C}$ . in air with homogenate from 0.5 g. rat liver suspended in 0.075 M. potassium phosphate buffer. The contents of the flasks were adjusted to pH 8.8 prior to incubation. The  $\text{O}_2$  uptake was 3.1  $\mu\text{M}$ .

Products, as Percentage of Incubated $-\text{C}^{14}\text{H}_3$	
$\text{CO}_2$	0.01
$\text{CH}_2\text{O}$	1.6
Dimethylaminoethanol	68.0

sufficient to oxidize approximately 25 per cent of the incubated  $\text{C}^{14}\text{H}_3$  to the level of formaldehyde.

*Methanol.* The failure of formaldehyde to accumulate when methanol was incubated with the washed liver sediment eliminates methanol as an important intermediate in the oxidation of dimethylglycine and sarcosine to formaldehyde in this system. Concerning the oxidation of methanol by the supernatant fraction (see Table 6), it may be mentioned that the addition of DL-methionine doubles the yield of formaldehyde. Allowing the supernatant to age for 24 hours in the refrigerator also increases the accumulation of formaldehyde, and adding methionine to such an aged preparation increases the formaldehyde accumulation 10-fold (25).

*Dimethylglycine and sarcosine.* Balance studies on the conversion of dimethylglycine and sarcosine to formaldehyde were carried out with intracellular components of liver fractionated in 0.25 M.

sucrose by the method of Schneider and Hogeboom (26). As shown in Tables 8 and 9, formaldehyde accumulated when either substrate

TABLE 8

ISOLATION OF FORMALDEHYDE FROM SARCOSINE (225  $\mu$ M.) INCUBATED WITH VARIOUS COMPONENTS OF LIVER CELLS

Fraction *	Mg. Formaldemethone
Homogenate	3.0
Nuclei †	2.1
Mitochondria	6.8
Supernatant	0
Reconstituted	3.0

\* Equivalent to 2.5 g. liver.

† No HCHO obtained with purified nuclear fraction.

was incubated with the mitochondria. The supernatant fraction containing the microsomes was not only inactive, but when mixed with the mitochondria substantially reduced the yield of formaldehyde.

TABLE 9

ISOLATION OF FORMALDEHYDE FROM DIMETHYLGLYCINE (225  $\mu$ M.) INCUBATED WITH VARIOUS COMPONENTS OF LIVER CELLS

Fraction *	Mg. Formaldemethone
Homogenate	2.2
Nuclei †	0.4
Mitochondria	1.8
Supernatant	0
Reconstituted	2.2

\* Equivalent to 2.5 g. liver.

† No HCHO obtained with purified nuclear fraction.

Presumably the latter result was due to the suppression of formaldehyde production or, alternatively, and more probably, to its increased participation in synthetic or oxidative reactions. When the several cellular fractions were reassembled the yield of formaldehyde was in good agreement with that obtained with the original homogenate, a fact indicating that the enzyme systems responsible for formaldehyde production from dimethylglycine and sarcosine are located in the mitochondria.



The activity of the nuclear fraction, which is heavily contaminated with mitochondria, deserves comment in two respects. First, nuclei prepared by the more refined procedure of Hogeboom, Schneider, and Striebich (27) and essentially free from mitochondria, do not produce formaldehyde from sarcosine or dimethylglycine. Second, the ratio of formaldehyde accumulation in the impure nuclear fraction to formaldehyde accumulation in mitochondria was much higher for sarcosine than for dimethylglycine (compare Tables 8 and 9), indicating that liver mitochondria are not homogenous with respect to size and, as a corollary, that sarcosine and dimethylglycine are oxidized by different enzymes (21).

#### SARCOSINE, DIMETHYLGLYCINE, AND A ONE-CARBON CYCLE

At the first opportunity the nature of the unknown metabolite formed in the oxidation of sarcosine by washed liver homogenates was investigated in collaboration with Sallach by the use of the paper chromatography technique. In confirmation of the report of Mitoma and Greenberg (28), it was found that large amounts of serine are produced in this system. Glycine also accumulated in about equal quantities. When methyl-labeled sarcosine<sup>2</sup> was employed, the

TABLE 10

SYNTHESIS OF  $\beta$ -C<sup>14</sup>-SERINE FROM  $\text{—C}^{14}\text{H}_3$  SARCOSINE IN LIVER SEDIMENT

	% incubated C <sup>14</sup> H <sub>3</sub>
CH <sub>2</sub> O	47.4
HCOOH	2.4
CO <sub>2</sub>	0.3
Protein	1.0
Serine	
<i>p</i> -OH-azobenz. sulfon.	51.7
free	52.5
toluenesulfonyl	51.1
$\beta$ -carbon	52.6
$\text{—COOH}$	0

<sup>2</sup> We are indebted to Dr. William H. Horner and Professor Vincent du Vigneaud for the methyl-labeled sarcosine used in this experiment.

serine isolated from the reaction mixture by the carrier technique (Table 10) accounted for approximately 50 per cent of the oxidized methyl carbon (calculated from the oxygen uptake). The serine, which was labeled exclusively in the  $\beta$ -carbon, plus the radio-formaldehyde were equivalent to approximately 97 per cent of the oxidized methyl carbon (29).

When dimethylglycine was incubated in similar preparations, chromatographic examination of the supernatant fraction revealed that, in addition to formaldehyde, serine and glycine were also formed in its metabolism (21). This observation raised two provo-

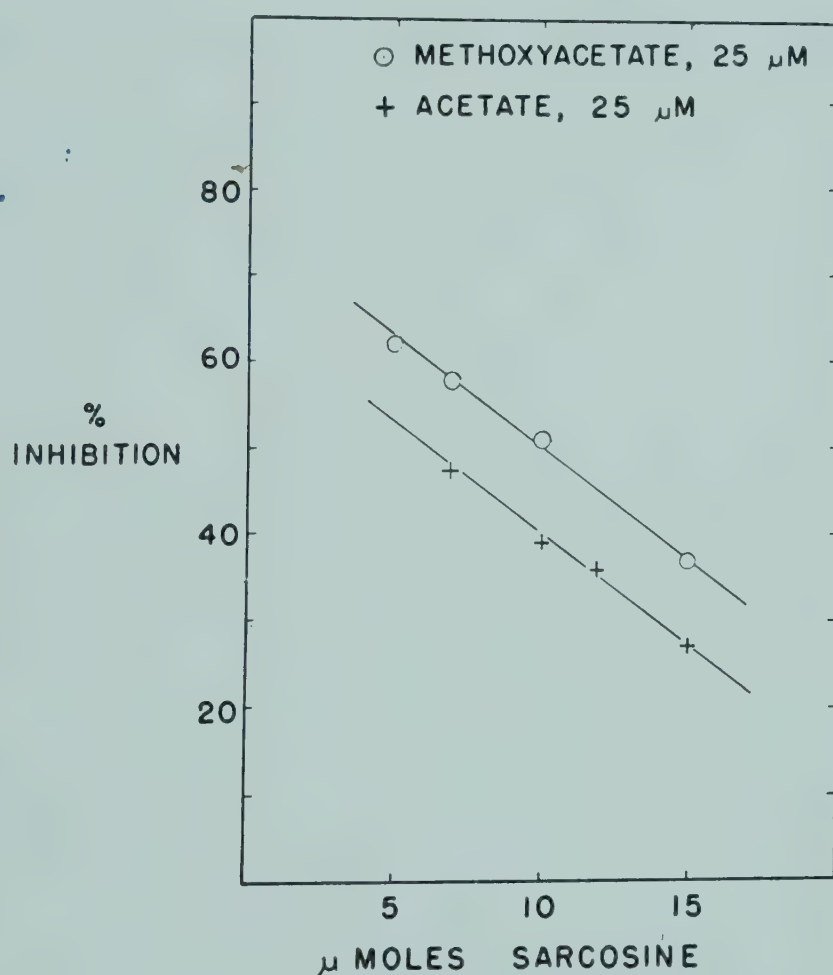


Fig. 1. Inhibition of sarcosine oxidase by methoxyacetate and by acetate.

cative and related questions: first, is dimethylglycine a precursor of sarcosine, and second, is dimethylglycine oxidized by sarcosine oxidase or by a specific dimethylglycine oxidase, as had already been suggested by the earlier experiments on the localization of formaldehyde-producing enzymes? It seemed likely that both of these questions might be answered if we could find a specific inhibitor of sarcosine oxidase. Methoxyacetate, because of its close structural



resemblance to sarcosine, was selected as a compound that might possess this property, and when tested this proved to be the case (21). As shown in Fig. 1, both methoxyacetate and acetate, which also was found to be an inhibitor of sarcosine oxidase, function competitively (30). When tested in parallel experiments in which formaldehyde was isolated directly from sarcosine and dimethylglycine (21), both inhibitors substantially reduced the quantity of formaldehyde isolated from sarcosine, whereas neither of them reduced the yield of formaldehyde from dimethylglycine (Table 11).

TABLE 11

INHIBITION OF SARCOSINE OXIDASE BY ACETATE AND METHOXYACETATE

Inhibitor 1.125 mM.	Mg. Formaldemethone	
	Sarcosine 0.225 mM.	Dimethylglycine 0.225 mM.
None	6.2	2.2
Methoxyacetate	1.1	2.3
Acetate	3.0	3.5

At this juncture in our studies, specific photometric methods for the determination of small quantities of serine and formaldehyde were developed in collaboration with Frisell and Meech (31). The methods gave values for the formaldehyde and serine formed in the oxidation of radiomethyl sarcosine that were in excellent agreement with those obtained by carrier isolation of radioformaldehyde and radioserine. Accordingly, the photometric procedures were applied to an examination of the effect of methoxyacetate on serine synthesis and formaldehyde accumulation from sarcosine and dimethylglycine. As shown in Table 12, when sarcosine was the substrate, serine formation as well as formaldehyde formation in washed rat liver mitochondria was inhibited by methoxyacetate, and the degree of inhibition was of the same magnitude for both reaction products. On the other hand, with dimethylglycine as the substrate (Table 13), the synthesis of serine was almost completely blocked, while there was very little depression in formaldehyde accumulation (32). Moreover, the oxygen uptake in the presence of the inhibitor was

TABLE 12

INHIBITION BY METHOXYACETATE OF SARCOSINE OXIDATION IN MITOCHONDRIA

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
5 $\mu$ M. Sarcosine	5.1	2.0	2.1
5 $\mu$ M. Sarcosine + 100 $\mu$ M. MOA	1.5	0.6	0.7
% Inhibition	70	70	67

TABLE 13

EFFECT OF METHOXYACETATE ON DIMETHYLGLYCINE METABOLISM IN MITOCHONDRIA

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
5 $\mu$ M. Dimethylglycine	5.4	2.2	1.0
5 $\mu$ M. DMG + 100 $\mu$ M. MOA	2.7	1.9	0.1
% Inhibition	50	13	90

reduced to about one-half of that observed with dimethylglycine alone. These experiments suggested that dimethylglycine was a precursor of sarcosine and that its conversion to serine was exclusively by way of sarcosine. Direct evidence that this is the case was obtained by incubating dimethylglycine with methoxyacetate, or acetate, and chromatographing the products of the reaction. When dimethylglycine was incubated with mitochondria alone, spots exhibiting the  $R_f$  values of serine and glycine were obtained. In the presence of methoxyacetate these spots were eliminated, and a strong spot with the  $R_f$  of sarcosine appeared (30, 32).

The results of these experiments (32), which are formulated in Fig. 2, indicate that dimethylglycine is oxidized to formaldehyde and sarcosine by a dimethylglycine oxidase located in the mitochondria. Sarcosine, in turn, is oxidized to glycine and formaldehyde. In the course of this oxidation a one-carbon compound condenses with glycine to yield serine. Thus both of the methyl groups of dimethylglycine are sources of formaldehyde, one by direct oxidation, and the other by way of sarcosine. It will be



recalled that Dubnoff (33) has found that choline is oxidized to betaine prior to the transmethylation reaction which results in the synthesis of methionine from homocysteine; and Muntz (34) has demonstrated that dimethylglycine is formed in the process. Thus,

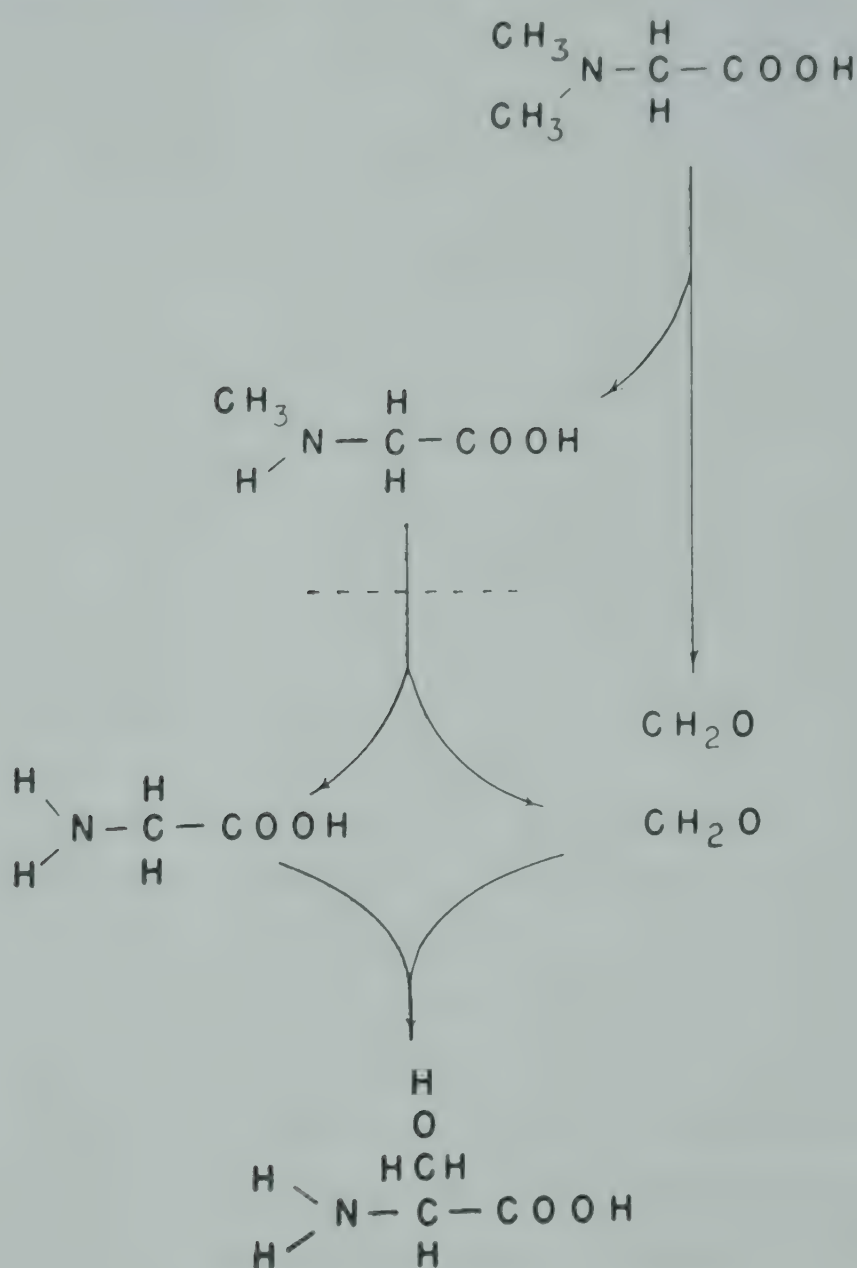


Fig. 2. Scheme for the conversion of dimethylglycine to sarcosine and thence via glycine to serine.

the dimethylglycine-sarcosine-glycine-serine series of reactions presented in Fig. 2 provides a pathway for the oxidation of two of the methyl groups of choline or betaine to formaldehyde, formate, and carbon dioxide. Consistent with such a pathway is our earlier observation that the methyl groups of betaine are sources of the methyl carbon of sarcosine in the intact animal (13). Moreover, Stetten (35), du Vigneaud, Simmonds, Chandler and Cohn (36), and

Soloway and Stetten (37) have shown that choline, betaine, and dimethylaminoethanol as well, are all sources of glycine in the rat. It will be recalled from a previous section of this paper that mitochondria and washed liver sediment, although potent sources of dimethylglycine and sarcosine oxidase, are devoid of activity with respect to the oxidation of the methyl carbons of choline, betaine, and methionine (21). Furthermore, as was shown in Table 1, the conversion of the methyl groups of choline and betaine to carbon dioxide in intact liver slices is very small compared to the rate of oxidation of the methyl carbon of sarcosine, an observation entirely consistent with an oxidative demethylation via sarcosine. Consequently, from an overall point of view there seems to be little doubt that dimethylglycine and sarcosine represent a major pathway for the oxidation of N-methyl groups in the body. In this connection, a comparison of the rate of oxidation of the methyl groups of dimethylglycine with those of betaine, both in isolated systems and in the intact animal, is a matter of considerable importance.

Dimethylglycine oxidase and sarcosine oxidase, as measured both by oxygen uptake, and by formaldehyde and serine formation, have been found to be present in the livers of the rat, hamster, guinea pig, and rabbit (32). Calculated on the basis of mitochondrial nitrogen, the initial rate of oxygen uptake with dimethylglycine as a substrate is about one-quarter of the rate observed with sarcosine. In contradistinction to the above species, chicken liver mitochondria were found to contain very little dimethylglycine or sarcosine oxidase.<sup>3</sup>

Since Stetten (39) and Arnstein (40) have shown that serine is converted to ethanolamine and du Vigneaud and his collaborators (23) have shown that monomethylethanolamine and dimethylethanolamine are precursors of choline in the rat, the reaction sequence illustrated in Fig. 2 completes a cycle for the oxidation of methyl groups and the generation of one-carbon compounds. In

<sup>3</sup> Mention should be made of the pioneer work of Jukes (38) on transmethylation in the chick, work which revealed among other things that, unlike the rat, the chick cannot use ethanolamine as a source of choline even in the presence of an abundant supply of methionine.



this cycle,<sup>4</sup> which is illustrated in Fig. 3, the N-methyl glycines and aminoethanols function as carriers. The cycle provides for the oxidation of methyl groups in two ways, either directly through single carbon compounds, or alternatively, by way of a more circuitous route involving decarboxylation. Starting at the top with betaine, it may be seen that following the loss of a methyl group in transmethylation, the two remaining methyl groups are oxidized to

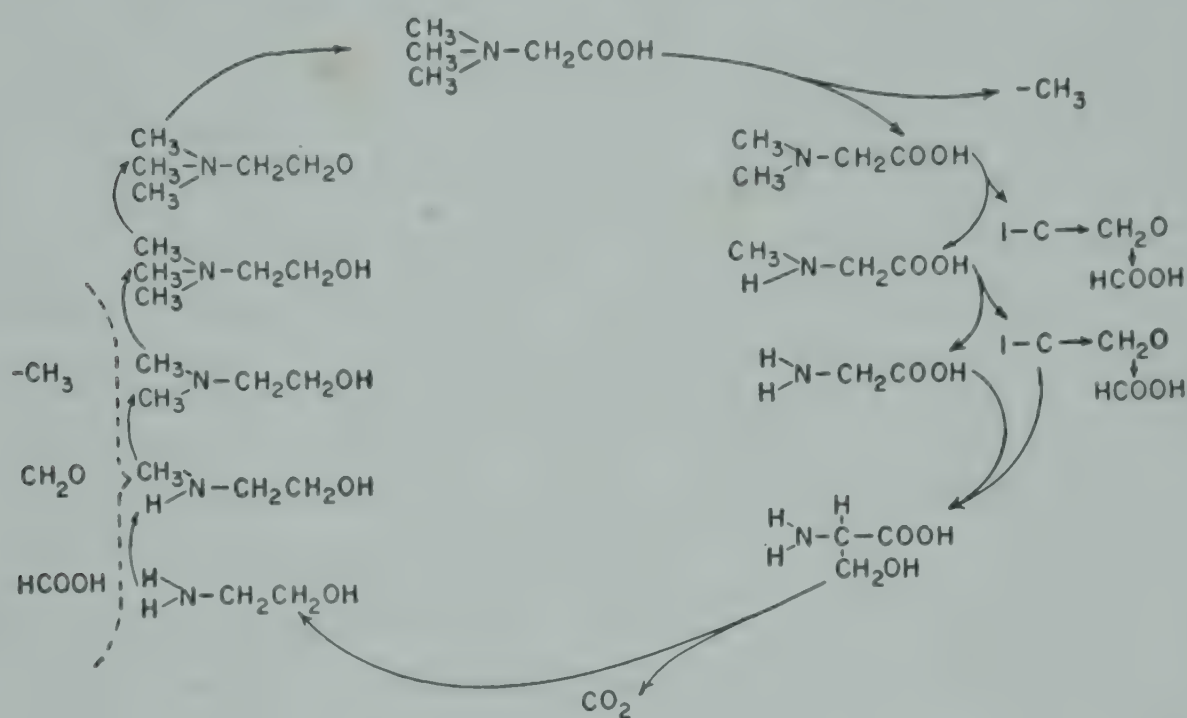


Fig. 3. The methyl oxidation cycle and the generation of one-carbon compounds.

formaldehyde, and thence to formate and carbon dioxide; or alternatively, a formaldehyde-like compound indicated by 1-C, may condense with the glycine formed in sarcosine oxidation to give the  $\beta$ -carbon of serine. The carboxyl group of this serine, derived originally from betaine, is then converted to carbon dioxide, leaving an erstwhile methyl carbon in the alcohol position of ethanolamine. The ethanolamine may then be methylated by methionine, as shown in the now classical experiments of du Vigneaud and his collaborators (16), and possibly by the products of methyl oxidation themselves,<sup>5</sup>

<sup>4</sup> A similar cycle, omitting dimethylglycine and sarcosine, was presented by Jukes (38) in 1947. Du Vigneaud (16) has formulated a similar scheme. However, neither of these included the formation of one-carbon compounds.

<sup>5</sup> It is not within the province of this paper to discuss the synthesis of labile methyl groups. Suffice to say that the experiments in vitro of Sakami and Welch,

to yield choline, which in turn is converted to betaine. It will be noted that the  $\beta$ -carbon of serine, which is derived from the 1-C compound, has now become the carboxyl carbon of betaine. Thus the carboxyl carbon of betaine may arise from the methyl group of betaine itself via dimethylglycine, sarcosine, a one-carbon compound, the  $\beta$ -carbon of serine, and the 1-carbon of the aminoethanols. In another revolution of the cycle this carbon is converted to carbon dioxide by the decarboxylation of serine.

It will be realized that this series of reactions represents only the skeleton, in my opinion only the backbone, of a much more complicated system of metabolic interrelationships. Shemin (44) showed in 1946 that serine is converted to glycine in the body without the loss of nitrogen, an observation recently confirmed and extended by Elwyn and Sprinson (45) and by Arnstein (46). Moreover, Weisbach, Elwyn, and Sprinson (47) have shown that the  $\beta$ -carbon of serine is a source of the methyl groups of choline and the methyl group of thymine. Arnstein (40) has also found that serine  $\beta$ -carbon is a source of the methyl groups of choline. Thus it would appear that the synthesis of serine indicated in Fig. 3 is reversible and that glycine and formaldehyde, or some other one-carbon compound, are regenerated in the backward reaction. Finally, it has been shown by the investigations of Siekevitz, Winnick, and Greenberg (18) and Sakami (19) that the  $\alpha$ -carbon of glycine itself can give rise to the  $\beta$ -carbon of serine. However, to the best of my knowledge this

the concomitant experiments in vivo of du Vigneaud and coworkers, and the work of Stekol and his collaborators have demonstrated the biological reversal of the oxidation of methyl groups. These experiments have been reviewed by du Vigneaud (16). The experiments of Berg (41) with a pigeon liver enzyme system and those of Arnstein and Neuberger (42) employing the intact rat indicate that the methyl group of methionine may be synthesized without choline as an intermediate. As shown by du Vigneaud and collaborators (43), essentially all of the methyl groups of tissue choline may be derived from dietary methionine. Whether the methyl groups of choline are ever directly synthesized, without methionine as an intermediate, is at present unknown. The organic synthesis of dimethylaminoethanol from formaldehyde and monomethylaminoethanol, with formate as a reducing agent, as described earlier in this paper (24), presents an excellent organic analogy for direct synthesis of the methyl groups of the N-methyl aminoethanols. Perhaps only the addition of the third methyl group to yield choline is restricted to a transmethylation reaction with methionine as the methyl donor.



reaction does not approach in efficiency the conversion of sarcosine to serine in yields of up to 70 per cent. Evidence has already been presented from our own work that, compared to sarcosine, glycine is a poor source of urinary formate (10). Obviously, to add to the cycle shown in Fig. 3 all of the reactions that have been just mentioned would lead to a picture so complicated that it would be difficult to follow. Indeed, the interrelationships and complexities in this scheme illustrate, perhaps more forcefully than any other series of relationships, the intermeshing of the reactions of intermediary metabolism and their seemingly endless ramifications that constitute the steady state and make it the resilient chemical homeostatic mechanism that it is.

Whether or not methyl groups are ever oxidized by a reversal of the reactions (methylation of aminoethanols) depicted at the left of Fig. 3 is at present not known. With respect to monomethylaminoethanol and choline, and indeed betaine, there is no evidence that this is the case. None of these compounds yields formaldehyde when incubated with homogenized liver. Dimethylaminoethanol, on the other hand, has been shown to yield formaldehyde in whole liver homogenates (21, 24). Presumably, it is either oxidatively demethylated directly to yield formaldehyde and monomethylaminoethanol, or it is oxidized to dimethylglycine and thence to formaldehyde. In the latter event this would constitute a shunt across the cycle and further complicate the picture of one-carbon metabolism.

With respect to the oxidation of the methyl group of methionine, which is indicated at the upper right in Fig. 3, it is clear from the work of du Vigneaud and his colleagues (16) that it may be incorporated into choline and hence oxidized via betaine, dimethylglycine, and sarcosine. Indeed, we showed that following the feeding of a large dose of methyl-labeled methionine the specific activity of both lipid-soluble and free choline exceeded the specific activity of the protein-bound methionine (7). It has also been demonstrated in collaboration with Horner (13) that the methyl group of methionine appears in the methyl carbon of sarcosine in the whole animal. Whether this incorporation proceeds solely by way of betaine (and



choline) or whether it also occurs as the result of the direct synthesis of sarcosine from glycine and a one-carbon entity is a matter of considerable interest.

In addition to the above pathways there is evidence that the methyl group of methionine is oxidized by more direct routes, for, as shown in Table 1, the rate of oxidation to carbon dioxide of the methyl group of methionine by liver and kidney slices is much faster than the rate of oxidation of the methyl groups of choline and betaine. However, I could never to my satisfaction isolate formaldehyde containing a significant degree of activity from liver slices following the incubation of methyl-labeled methionine (25), although with radiosarcosine as the substrate, radioformaldehyde was consistently obtained (10). This suggests that methionine methyl may be oxidized, either directly or following transmethylation, by a pathway which does not involve formaldehyde. Consequently we cannot say that the cycle depicted in Fig. 3 presents the sole pathway for the metabolism of the methyl group of methionine. Nor indeed can this statement be made with respect to any of the compounds in question. However, it appears to the writer that the scheme depicted in Figs. 2 and 3 represents a major and important pathway, not only for the conversion of methyl groups to carbon dioxide, but also for the generation of considerable quantities of one-carbon compounds, which, by their extensive participation in synthetic reactions, earns for many of the methyl carbons a brief respite from their ultimate metabolic fate, the formation of carbon dioxide and water.

#### OXIDATIVE REARRANGEMENT OF SARCOSINE AND FORMATION OF ACTIVE FORMALDEHYDE

*The rearrangement of sarcosine.* The key position occupied by sarcosine in the foregoing series of reactions and the large yields of serine obtained from it, as well as the belief that its metabolism represented an excellent spectrum of biochemical reactions for the study of integrated enzyme systems, such as those in mitochondria, prompted us to examine in some detail the mechanism whereby it is converted to serine. In the isotope experiments carried out in



collaboration with Sallach (32), radiosarcosine was incubated for two hours with the washed mitochondria obtained from 0.5 g. of rat liver. The serine and formaldehyde content of an aliquot of the reaction mixture was measured by our photometric procedures (31), and, at the same time, formaldehyde and serine were isolated by the carrier technique. The serine was counted as the para-OH-azobenzenesulfonate, as free serine, and finally as the tosyl derivative. From the quantitative determination of radioserine and formaldehyde, together with the photometric determination of these two compounds, the "absolute" specific activities of the serine and formaldehyde formed in the reaction mixture were calculated and compared with the specific activity of the starting sarcosine. The free serine was degraded by periodate oxidation and the number-one and number-three ( $\beta$ ) carbons were isolated respectively as barium carbonate and the dimedon derivative of formaldehyde. In all single-label experiments the activity of one or the other of the degradation products corresponded to the total activity of the free serine. Comparison was then made between the individual carbon atoms of the synthesized serine and the carbons of the starting sarcosine.

In the reactions given below, the micromoles of substrate and products are given above the formulas. The figures beneath the formulas are the specific activities, in terms of the starting material, of the individual carbon atoms. All activities were corrected for self-absorption and are expressed as counts per minute per micromole. In all of the experiments approximately one microatom of oxygen was taken up per micromole of sarcosine incubated.

The initial experiments were made with methyl-labeled sarcosine.<sup>2</sup>  $C^{14}$  was found in the formaldehyde and serine isolated from the TCA filtrate of the mitochondria at the end of the two-hour incubation period. As shown in Fig. 4, all of the activity of the serine was in the  $\beta$ -carbon. Both the serine  $\beta$ -carbon and the free formaldehyde possessed the same activity as the methyl carbon of the starting sarcosine. In the next experiments carboxyl-labeled sarcosine synthesized by Mr. Abeles in our laboratory was employed as the substrate. The formaldehyde was devoid of activity, while the serine

formed had the same specific activity as the starting sarcosine. All of the activity of the isolated serine was located in the carboxyl carbon (Fig. 5).

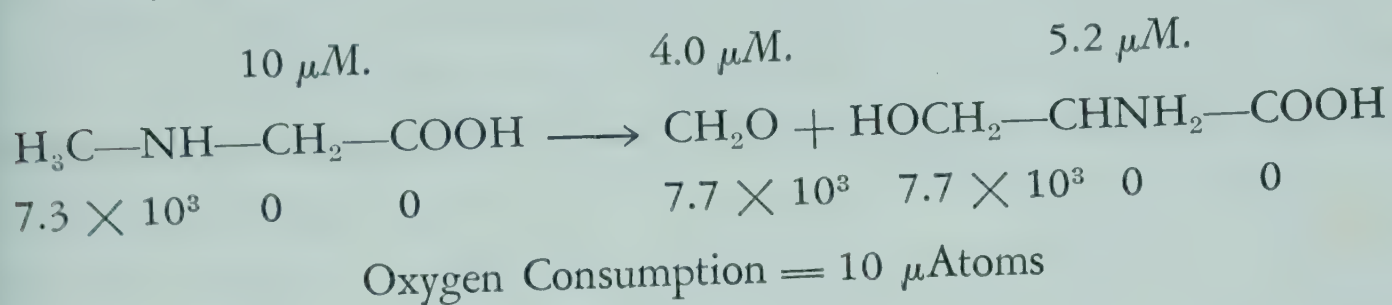


Fig. 4. Conversion of  $-\text{C}^{14}\text{H}_3$  sarcosine to formaldehyde and serine in mitochondria.

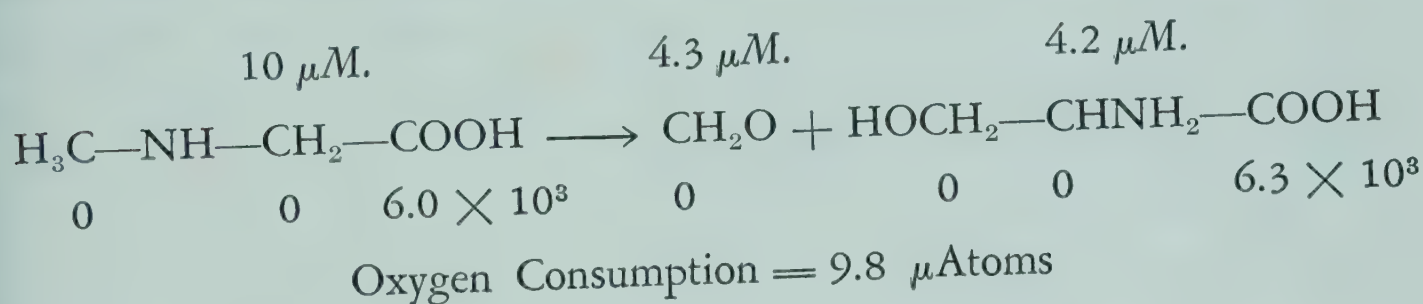


Fig. 5. Conversion of  $-\text{C}^{14}\text{OOH}$  sarcosine to formaldehyde and serine in mitochondria.

When a mixture of sarcosine labeled in the methyl and carboxyl carbons was employed, the ratio of  $\text{C}^{14}$  in the carboxyl and  $\beta$ -carbons of serine was the same as the ratio of  $\text{C}^{14}$  in the carboxyl and methyl carbons of the starting sarcosine (Fig. 6). Moreover, the specific

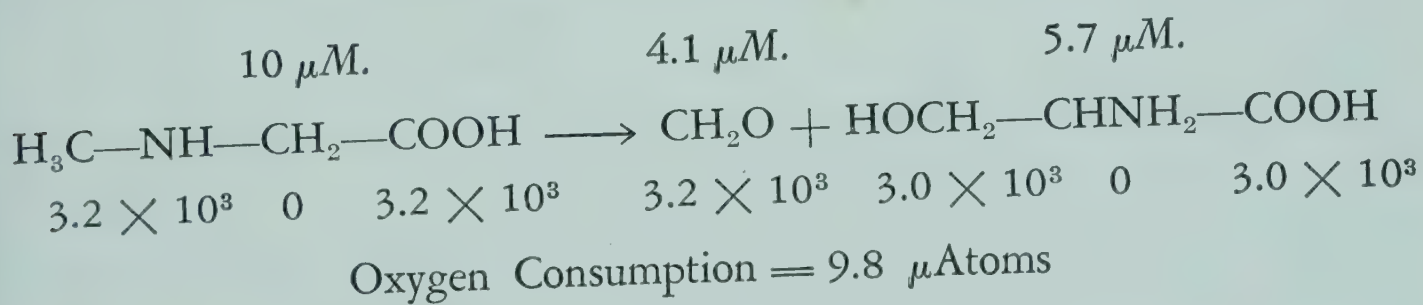


Fig. 6. Conversion of  $-\text{C}^{14}\text{OOH}$ ,  $-\text{C}^{14}\text{H}_3$  sarcosine to formaldehyde and serine in mitochondria.

activities of the serine carbons were in good agreement with the specific activities of the corresponding carbons of the sarcosine. In this, as in the foregoing experiments, the activity of the degradation products of serine was in such good agreement with the activity of the whole serine molecule that it could be concluded with safety that the serine  $\alpha$ -carbon was devoid of radioactivity.



These experiments demonstrated that in our mitochondrial preparation the  $\beta$ -carbon of serine is derived exclusively from the methyl carbon of sarcosine, and the carboxyl and  $\alpha$ -carbons of serine are derived exclusively from their counterparts in the glycine moiety of sarcosine. In this system there are no significant endogenous sources of serine, glycine, formaldehyde, or serine  $\beta$ -carbon. Moreover, the results showed that the  $\alpha$ -carbon of the glycine moiety of sarcosine was not a significant source of the  $\beta$ -carbon of serine under the conditions of our study.<sup>6</sup> In other words, sarcosine in the presence of mitochondria undergoes an oxidative rearrangement to yield serine and formaldehyde. When subjected to the action of a purified D-amino acid oxidase by Frisell and myself (49), this serine was found to possess the L-configuration.

It had been reported by Mitoma and Greenberg (28) and confirmed by us that the addition of exogenous glycine to the reaction mixture increased the yield of serine and reduced the accumulation of formaldehyde. It seemed advisable to determine whether or not the added glycine participated directly in the synthesis of the serine, or alternatively, whether its effect was on the serine synthesizing enzyme, etc. Accordingly, methyl-labeled sarcosine was incubated with carboxyl-labeled glycine. The yield of  $\beta$ -labeled serine was markedly increased, and the carboxyl carbon was labeled with  $C^{14}$  derived from the carboxyl group of glycine, 44 per cent of the radioglycine having entered the serine molecule. The results are shown in Fig. 7, where, in this instance, the yields are expressed as percentages of the starting  $C^{14}$ . Thus, exogenous glycine behaved like the glycine formed in the oxidation of sarcosine.<sup>7</sup>

It may be mentioned that in the mitochondrial system used in these experiments added serine does not disappear during a two-hour

<sup>6</sup> When carboxyl-labeled glycine was incubated with mitochondria alone, approximately 2 per cent was converted to carboxyl-labeled serine.

<sup>7</sup> Quantitatively speaking, the glycine derived from sarcosine appeared to be a more potent source of the number 1 and 2 carbons of serine than did the exogenous glycine. In other words, glycine produced in the mitochondria by sarcosine oxidase was more available to the contiguous serine-synthesizing enzyme than exogenous glycine, even though the initial concentration of the latter was much higher in the incubation mixture.

incubation period and can be recovered quantitatively from the trichloroacetic acid supernatant fraction. When formate or formaldehyde are added to the washed mitochondria there is no increase in the oxygen uptake. However, approximately 20 per cent of the added formaldehyde is bound by the protein (TCA-insoluble) fraction, from which it may be recovered by suspending in dilute phosphoric acid and distilling.

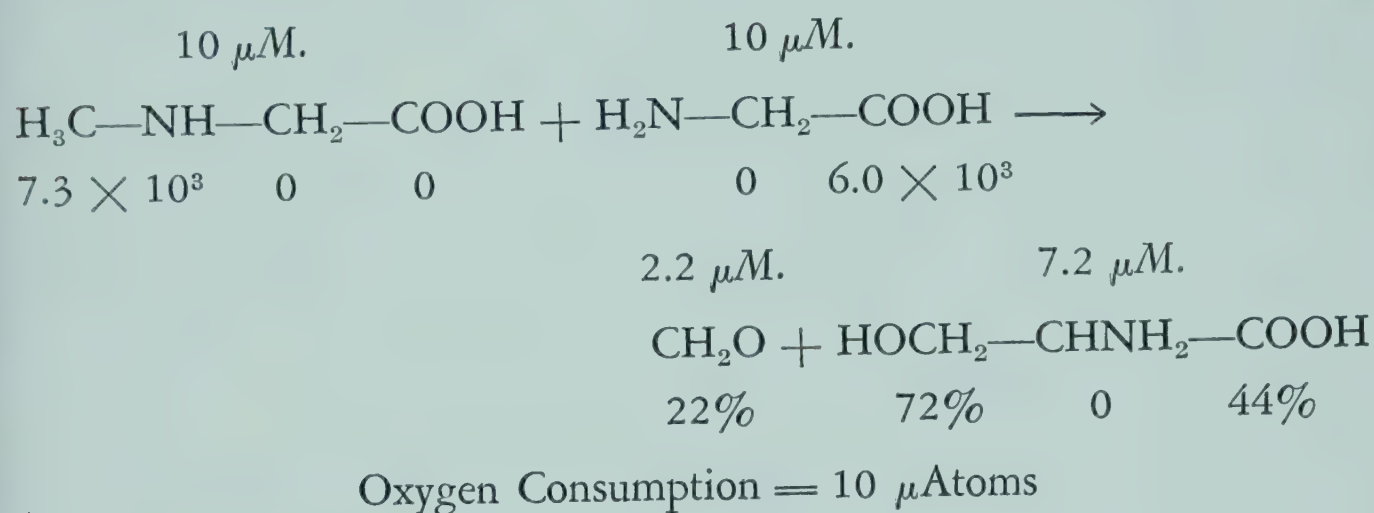


Fig. 7. Participation of glycine in the synthesis of serine from sarcosine in mitochondria.

*The active one-carbon compound from sarcosine.* With the establishment of the facts enumerated above, it was possible to examine with considerable accuracy the roles of formaldehyde and formate in the conversion of sarcosine to serine. To this end, 5  $\mu$ M. of non-isotopic formaldehyde (higher levels begin to inhibit serine synthesis) were added to a mitochondrial preparation that was actively converting 10  $\mu$ M. of methyl-labeled sarcosine to serine. When the serine was isolated and the specific activity of the  $\beta$ -carbon determined, it was found to be identical with that of the methyl carbon of the starting sarcosine. In other words, dilution by the nonisotopic formaldehyde had not occurred within the limits of our analytical procedures.

The experiment was then reversed and its sensitivity increased by adding radioactive formaldehyde to carboxyl-labeled sarcosine in the presence of mitochondria. The specific activity of the serine synthesized in this preparation was identical with the activity of the



carboxyl-labeled sarcosine. Furthermore, as shown in Fig. 8, all of the activity of the isolated serine was contained in the carboxyl carbon, the  $\beta$ -carbon being devoid of detectable quantities of  $C^{14}$ . Moreover, the added radioformaldehyde was quantitatively recovered from the trichloroacetic acid filtrate plus the protein fraction of the incubation mixture. These experiments demonstrate that formalde-

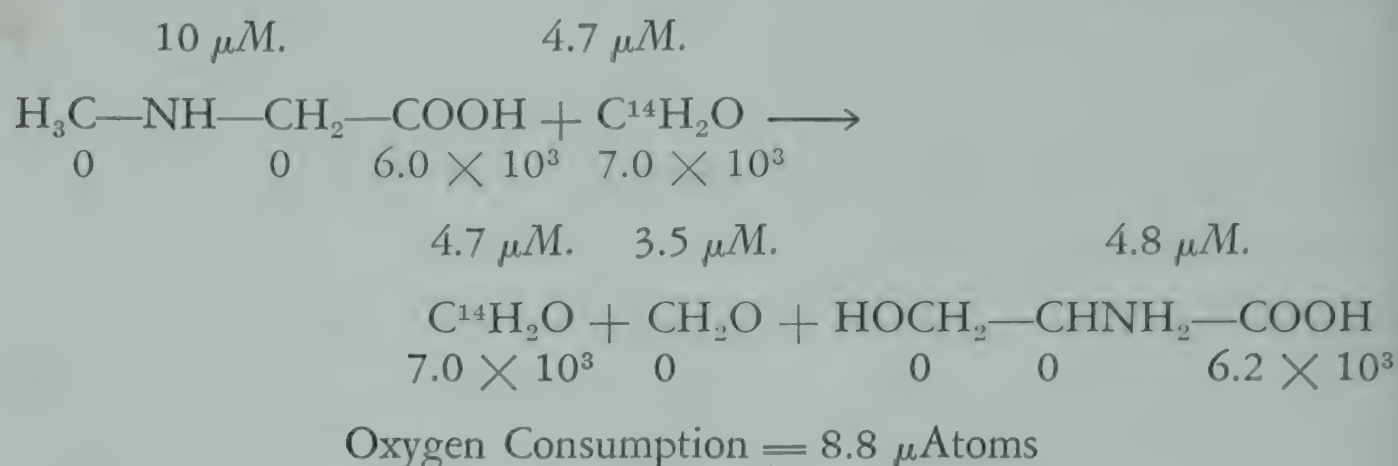


Fig. 8. Synthesis of  $-\text{C}^{14}\text{OOH}$  serine from  $-\text{C}^{14}\text{OOH}$  sarcosine in the presence of  $\text{C}^{14}\text{H}_2\text{O}$ .

hyde is not the one-carbon compound which condenses with glycine to yield serine. Furthermore, they demonstrate that, contrary to Mitoma and Greenberg (28), formaldehyde is not an intermediate on the metabolic pathway of the conversion of the methyl carbon of sarcosine to the  $\beta$ -carbon of serine. We have obtained similar results with radioformate.

These experiments with Sallach (32), which are formulated in Fig. 9, indicate that glycine and an active one-carbon entity are formed in the oxidative demethylation of sarcosine. This one-carbon compound may then condense with glycine to yield serine, or alternatively, it may be converted irreversibly in mitochondria to formaldehyde. That the formaldehyde is not produced by an independent oxidative reaction differing from the one giving rise to the active one-carbon entity was indicated in the experiments utilizing exogenous glycine, where the synthesis of serine was increased and the formation of formaldehyde was decreased by the same increment. In contrast to this decrease in formaldehyde with increased synthesis of serine has been the finding, to be discussed later, that formalde-

hyde can be removed during the course of the reaction without affecting the yield of serine. Apparently then, the conversion of the one-carbon entity to formaldehyde is a first-order reaction that depends only on the concentration of the one-carbon entity.

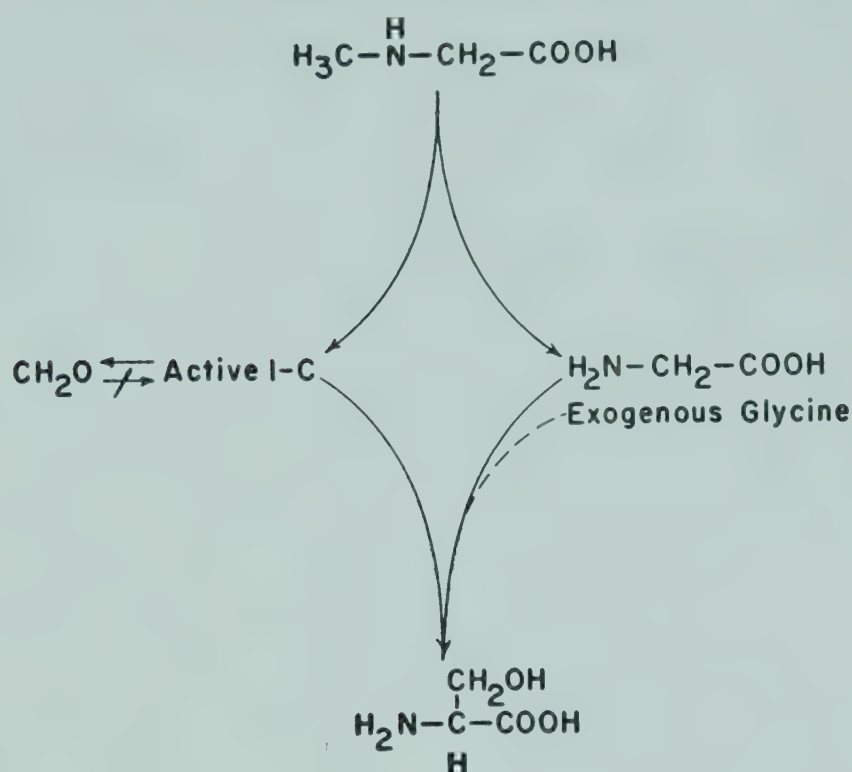


Figure 9. The formation of an active one-carbon compound and serine synthesis in the metabolism of sarcosine in mitochondria.

The active one-carbon compound depicted in Fig. 9 is not the aminolevulinic acid of Shemin (50), since the latter compound does not yield serine when incubated with glycine in the presence of mitochondria.<sup>8</sup> Furthermore, the active one-carbon compound formed from sarcosine is not identical with the formaldehyde-homocysteine mixture employed by Berg (41) in the synthesis of serine by pigeon liver enzymes, for such a mixture plus glycine does not yield serine in the presence of rat liver mitochondria. Also the failure of formaldehyde plus glycine to yield serine in quantities comparable to those obtained from sarcosine indicates that our active one-carbon compound is not the free tetrahydrofolic acid derivative implicated by Kisliuk and Sakami (51) and simultaneously by Blakeley, *Nature* 173, 729 (1954), in serine synthesis. The term "active one-carbon compound" is used without prejudice and by

<sup>8</sup> The aminolevulinic acid was kindly supplied by Professor David Shemin.



analogy with the active two-carbon compound. Like the latter, the active one-carbon compound probably contains several carbon atoms. However, one of these carbons, derived from the methyl group of sarcosine, is active in synthetic reactions and as a source of formaldehyde. It may even be the initial dehydrogenation product of sarcosine; for example, a methylene-glycine-enzyme complex. Certainly one might expect a compound of this nature to be on the pathway of sarcosine oxidation. In this connection we have obtained a 10 per cent yield of serine, according to photometric determination, when mitochondria are incubated with a solution of glycine and formaldehyde prepared 24 hours earlier (29). This yield suggests that "free" methyleneglycine may be at best only a byproduct of the reactions leading directly to the formation of the one-carbon compound. What should be emphasized at the present time is the extremely high activity of this one-carbon entity. With Sprague-Dawley rats the conversion of sarcosine to serine, even without added glycine, is in the neighborhood of 60 per cent.<sup>9</sup>

*The active one-carbon compound from dimethylglycine.* In considering the structure, as well as the occurrence, of the one-carbon compound it was particularly pertinent to determine whether the "first methyl group" of dimethylglycine could give rise to this entity. By employing extra glycine as an acceptor for the active one-carbon compound, we (52) were able to obtain 4.3  $\mu M$ . of serine from 3  $\mu M$ . of dimethylglycine (Table 14). Glycine alone gave no detectable serine or formaldehyde. Since at the most only 3  $\mu M$ . of serine could have been formed from dimethylglycine via sarcosine, it appears that the first methyl group of dimethylglycine is also converted to an active one-carbon compound capable of condensation with glycine to yield serine. The results of this experiment indicate

<sup>9</sup> The foregoing discussion is not meant to exclude the possibility that the production of an active one-carbon compound from methyl groups in mitochondria, and the formation of an active one-carbon compound from formaldehyde and tetrahydrofolic acid in the pigeon liver preparation of Kisliuk and Sakami (51) involve the same enzymatic components. However, if this is the case, the structural organization and biochemical function of these components differ substantially in mitochondria as compared to the more dispersed system.



TABLE 14

PARTICIPATION OF THE METHYL GROUPS OF DIMETHYLGLYCINE IN SERINE SYNTHESIS

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
3 $\mu$ M. Dimethylglycine	5.8	2.5	1.6
3 $\mu$ M. DMG + 10 $\mu$ M. Glycine	6.2	0.8	4.3
10 $\mu$ M. Glycine	0.7	0	0

that two of the three methyl groups of betaine (and choline) are sources of an active one-carbon compound which is neither free formaldehyde nor formate, and which, in the system employed, is not derived from either of them. This conversion was indicated earlier in Fig. 3 by the term 1-C.

The formation of an active one-carbon compound (or formaldehyde) from a dimethylamine, such as dimethylglycine, raises interesting problems concerning the mechanism of oxidation of tertiary methyl groups. Two possibilities exist: first, that prior to oxidation the methyl group of dimethylglycine is transferred intact to some other compound, and for this there is no evidence (53); and second, and more probably, that the first methyl group is oxidized while attached to the parent molecule. This might proceed in one of several ways. Hydrogen might be removed from each of the methyl groups to form a di-free radical or a carbon-to-carbon bond between the two methyl groups. Alternatively, a proton may be added to the nitrogen of dimethylglycine, thus permitting the consecutive removal of two hydrogens from a carbon and nitrogen to give a double bond, which in turn can be attacked by water or some other agent. In more general terms, the latter hypothesis states that the addition of a proton to the unpaired electrons of a tertiary amine, or of a thio-ether such as methionine or S-methyl cysteine, is the first step in the mechanism whereby the methyl group of such compounds is dehydrogenated. From this point of view, the methyl groups of such compounds as betaine should not be susceptible to direct oxidation. So far the evidence suggests that they are not oxidized until after they have been converted to tertiary amines. Actually, the



quaternary amines and the sulfonium compounds appear to be concerned with methyl transfer (54), which is the exchange of a positively charged methyl group for a proton (or the exchange of a methyl radical for a hydrogen atom). Thus in the conversion of betaine to methionine one might expect a positively charged dimethylglycine to be the product, and this in turn could undergo oxidation directly to an active one-carbon compound and sarcosine.

*Active formaldehyde.* Since the demonstration by Sakami (14) that exogenous formate is a source of the  $\beta$ -carbon of serine in the intact animal, and the finding by Greenberg and his collaborators (17, 18) as well as Sakami (19) that the  $\alpha$ -carbon of glycine is also a source of the  $\beta$ -carbon of serine, the one-carbon compound involved in serine synthesis has been variously referred to as formate, "formate-like," "closer to formaldehyde than formate," etc. It was therefore of more than passing interest to determine the oxidation level of the one-carbon compound derived from sarcosine that gave such high yields of serine in the presence of liver mitochondria. By adding semicarbazide to mitochondria that were oxidizing sarcosine, Frisell and I (32) obtained evidence that this one-carbon compound possesses the oxidation level of formaldehyde. As shown in Table 15, all of the methyl carbon was trapped as "formaldehyde"

TABLE 15

ACTION OF SEMICARBAZIDE ON FORMALDEHYDE AND SERINE ACCUMULATION FROM SARCOSINE INCUBATED WITH MITOCHONDRIA

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
20 $\mu$ M. Sarcosine	14.8	6.4	6.5
20 $\mu$ M. Sarcosine + 50 $\mu$ M. Semicarbazide	13.1	13.4	0

and no serine was synthesized. Since, as mentioned earlier, the conversion of the one-carbon compound to formaldehyde appears to be a first-order reaction, this result suggests that the active one-carbon compound combines directly with semicarbazide, or alternatively, that semicarbazide interferes with its combination with a carrier.

In either event there is no appreciable change in the oxygen consumption due to the addition of semicarbazide and the trapping of the methyl carbon of sarcosine as formaldehyde.

More definitive evidence of the oxidation level of the active one-carbon compound was sought in collaboration with Abeles (55) by labeling the methyl hydrogens of sarcosine with deuterium. Employing completely deuterated methyl iodide and tosyl glycine, sarcosine was synthesized containing virtually 100 atoms per cent excess D in the methyl group. This point is of some importance, inasmuch as we have found that deuteriosarcosine is oxidized at a slower rate than protium sarcosine, and hence in a mixture of the two the oxidation product (trapped as formaldehyde) is enriched with respect to protium (55). However, when completely deuterated sarcosine is used, this difficulty is obviated, and the slower oxidation rate does not vitiate the results.

The sarcosine containing a completely deuterated methyl group was incubated with mitochondria in a large-scale experiment, and formaldehyde was isolated without carrier from the supernatant fraction of the reaction mixture. The dimedon derivative was recrystallized several times from alcohol and water, and dried to constant weight. The serine present in another aliquot of the reaction mixture was separated from glycine, sarcosine, and formaldehyde by column chromatography and the serine  $\beta$ -carbon was oxidized to formaldehyde with periodate. This formaldehyde was also isolated without carrier as the dimedon derivative. The deuterium content of

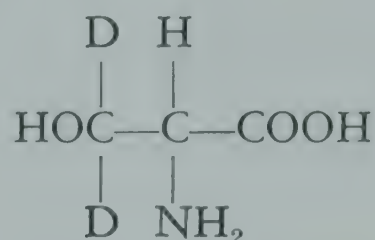
TABLE 16

DEUTERIUM CONTENT OF FORMALDEHYDE AND SERINE  $\beta$ -CARBON PRODUCED IN THE METABOLISM OF  $\text{CD}_3$  SARCOSINE

	Atom % Excess D	
	Found	Calculated for $>\text{CD}_2$ Transfer
Incubated sarcosine HCl	$36.2 \pm 0.7$	
Isolated formaldehyde (dimedon)	$8.06 \pm 0.7$	8.1
Isolated serine $\beta$ -carbon (dimedon)	$7.18 \pm 0.14$	8.1



the starting sarcosine, the metabolic formaldehyde, and the formaldehyde obtained from the  $\beta$ -carbon of serine was determined by Dr. Julian Rachele in du Vigneaud's laboratory. The results are shown in Table 16. Approximately 90 per cent of the hydrogen atoms found in the  $\beta$ -carbon of serine were present as deuterium. It may be seen from the calculated deuterium transfer that a minimum of 8 out of 10 of the serine molecules had the following composition:



These results provide convincing evidence that the active one-carbon compound possesses the oxidation level of formaldehyde. Furthermore, they show that in its transfer the carbon-bound hydrogen does not exchange extensively with the protons or hydrogen atoms of the environment. Since in our system formaldehyde is neither on the pathway of formation or transfer of this entity, the active one-carbon compound is, so to speak, on the other side of formaldehyde from formate. We have referred to this active one-carbon compound, which is also a precursor of formaldehyde, as "active formaldehyde." In the preceding figures active formaldehyde is indicated by "1-C." Even if a levulinic acid or a tetrahydrofolic acid derivative turns out to be a more *immediate* precursor of serine in our system, the highly active compound formed in the oxidation of dimethylglycine and sarcosine will still fulfill the definition for an "active formaldehyde," since it cannot be replaced by formaldehyde or formate. Consequently, active formaldehyde derived from the methyl groups of sarcosine and dimethylglycine, and ultimately from all biologically labile methyl compounds, appears to differ from other active one-carbon compounds. Whether the other one-carbon compounds, like formaldehyde, formate, the  $\beta$ -carbon of glycine, etc., which can act as sources of serine  $\beta$ -carbon in more complicated systems, must first be converted to formaldehyde is a question of importance.

The finding that the methyl groups may be converted via "active



formaldehyde" to the  $\beta$ -carbon of serine is of considerable interest when taken in conjunction with the finding of Elwyn, Weissbach, and Sprinson (56), on the conversion of the  $\beta$ -carbon of serine to the methyl groups of choline and thymine. Employing deuteroserine, these investigators showed in whole animal experiments that the  $\beta$ -carbon of serine does not lose or exchange hydrogens in its conversion to methyl groups. Thus, methyl groups may be oxidized to the level of formaldehyde and may be converted back to methyl groups without attaining the oxidation level of formate. Indeed, in view of our results, which demonstrate that methyl groups do not pass through free formaldehyde in their conversion to serine  $\beta$ -carbon, it is not improbable that this is also the case in the conversion of serine  $\beta$ -carbon to choline or thymine. Thus it may be that biologically labile methyl groups circulate via "active formaldehyde," as well as by transmethylation, without passing through formaldehyde and formate. At any stage in this inner cycle active formaldehyde may be converted to free formaldehyde, which possesses an entirely different synthetic potential with respect to biological reactions—for example, condensation with pyruvate, as will be shown later. Free formaldehyde can be converted in turn to formate, which is active in still other respects. Of course, the backward reactions from formate and formaldehyde to methyl groups may occur, as has been shown by du Vigneaud and his colleagues (16). The equilibria in these reactions are not known. However, one suspects that in the intact animal they are towards the less complex form and the higher oxidation level.

#### EFFECT OF COFACTORS ON SARCOSINE OXIDATION AND SERINE SYNTHESIS

In the experiments that have been described thus far no cofactors were added to the washed mitochondria. This preparation is complete in itself for the conversion of dimethylglycine to sarcosine and of sarcosine to serine, with the elaboration of active formaldehyde at each of these steps. However, several coenzymes have most interesting effects on the system.



*Diphosphopyridine nucleotide.* When DPN was added to the reaction mixture there was an increase in the oxygen uptake, and formaldehyde did not *accumulate*. As shown in Table 17, there was, however, no depression in serine synthesis. These results indicate that although free formaldehyde was oxidized to formate, the active

TABLE 17

ACTION OF DPN ON FORMALDEHYDE AND SERINE ACCUMULATION  
FROM SARCOSINE INCUBATED WITH MITOCHONDRIA

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
10 $\mu$ M. Sarcosine	10.0	4.2	4.7
10 $\mu$ M. Sarcosine + 2.4 $\mu$ M. DPN	16.0	0	5.1
5 $\mu$ M. CH <sub>2</sub> O	0	4.0	0
5 $\mu$ M. CH <sub>2</sub> O + 2.4 $\mu$ M. DPN	5.0	0	0

formaldehyde was not attacked by the coenzyme. Such a coenzyme specificity is in agreement with the radioisotope experiments, which showed that added formaldehyde does not participate in the conversion of sarcosine to serine. Moreover, we may conclude from the effect of added DPN that DPN is either not a coenzyme for sarcosine oxidase, or, if so, that it is present in a bound form that is inaccessible or unavailable for the oxidation of formaldehyde.

*Flavine adenine dinucleotide.* In addition to DPN, several other compounds had the interesting effect of preventing the accumulation of formaldehyde without substantially lowering the synthesis of serine. However, before discussing these compounds it seems appropriate to describe the action of another coenzyme, FAD, on the mitochondrial-sarcosine system. Mitochondria prepared from the livers of riboflavin-deficient rats oxidized sarcosine at a lower than normal rate, both as measured by oxygen consumption, and by the photometric determination of formaldehyde and serine. When FAD was added to the mitochondria prepared from the deficient animals, there was a two- or three-fold increase in oxygen consumption. However, in every instance restoration of oxygen consumption to (or above) the normal level was accompanied by a very marked

reduction in formaldehyde and serine. When FAD was added to the mitochondria obtained from *normal* animals, formaldehyde and serine accumulations were also drastically reduced. The increase in oxygen uptake was approximately that required to oxidize the methyl carbon to formate. In other experiments it was shown that the addition of FAD causes the oxidation of formaldehyde by washed rat liver mitochondria. Added L-serine, on the other hand, may be recovered from the same preparation after 2 hours incubation. It appears therefore that FAD, like DPN, acts as a coenzyme in the oxidation of formaldehyde by mitochondria. However, unlike DPN, it also catalyzes the oxidation of active formaldehyde.

It may be inferred from these experiments that FAD either is not involved in sarcosine oxidation or, if so, it is so tightly imbedded in the mitochondria as to be resistant to removal by repeated washings with buffer and to be inaccessible as an electron acceptor in the oxidation of formaldehyde or active formaldehyde. Moreover, it appears that the reduced rate of sarcosine oxidation by mitochondria from riboflavin-deficient rats may be an indirect effect of the deficiency on the composition or quantity of mitochondria in liver.

#### EFFECT OF ALANINE AND PYRUVATE ON SARCOSINE OXIDATION AND SERINE SYNTHESIS

It appeared probable that the synthesis of L-serine in the mitochondrial system required, in accordance with the theory of Ogston (57), a 3-point attachment of glycine to the serine-synthesizing enzyme. From this it was deduced that either D- or L-alanine should act as an inhibitor in serine synthesis, depending upon whether the replacement reaction between "active formaldehyde" and glycine required that the "L-hydrogen" of glycine be free or buried in the enzyme surface. It was found (25), as shown in Table 18, that D-alanine substantially reduced the accumulation of serine, whereas L-alanine was without effect. Since D-alanine caused an increase in the oxygen consumption, it seemed probable that pyruvate was being formed as a result of D-amino acid oxidase activity, and that this pyruvate, rather than the D-alanine, was responsible for the reduc-



TABLE 18

ACTION OF ALANINE ON FORMALDEHYDE AND SERINE ACCUMULATION  
FROM SARCOSINE INCUBATED WITH MITOCHONDRIA

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
10 $\mu$ M. Sarcosine	9.7	4.2	4.7
10 $\mu$ M. Sarcosine + 100 $\mu$ M. D-Alanine	12.7	4.4	1.9
10 $\mu$ M. Sarcosine + 100 $\mu$ M. L-Alanine	9.7	4.4	4.5

tion in serine synthesis. Mitchell and Artom (58) had previously reported that pyruvate caused the disappearance of formaldehyde from a washed liver sediment preparation. Hift and Mahler (59) had made the same observation and had solubilized and purified the enzyme responsible for this reaction. The reaction product was isolated and identified as  $\alpha$ -keto- $\gamma$ -hydroxybutyric acid. So far, evidence was lacking as to whether or not this enzyme could condense active formaldehyde and pyruvate, or, for that matter, whether it could synthesize serine from active formaldehyde and glycine.

TABLE 19

ACTION OF PYRUVATE ON FORMALDEHYDE AND SERINE ACCUMULATION  
FROM SARCOSINE INCUBATED WITH MITOCHONDRIA

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
10 $\mu$ M. Sarcosine	9.6	4.3	3.9
10 $\mu$ M. Sarcosine + 10 $\mu$ M. Pyruvate	9.6	1.8	4.2
5 $\mu$ M. CH <sub>2</sub> O	0	4.0	0
5 $\mu$ M. CH <sub>2</sub> O + 5 $\mu$ M. Pyruvate	0	1.8	0

In view of the above findings, sarcosine and pyruvate were incubated with liver mitochondria. The effect of pyruvate (Table 19) was just the opposite from that observed with D-alanine. There was no increase in oxygen uptake, and formaldehyde accumulation, rather than serine accumulation, was reduced. When formaldehyde and pyruvate were added to washed mitochondria alone, the formal-

dehyde disappeared in the absence of any oxygen uptake. Moreover, the reaction mixture contained no formallogenic compound when oxidized with periodate. This is in agreement with the structure of the formaldehyde-pyruvate condensation compound determined by Hift and Mahler. In view of these results, the inhibition of serine synthesis by D-alanine cannot be ascribed to the conversion of the latter compound to pyruvate. It has been shown that D-alanine does not interfere with the periodate determination of serine. Moreover, its effect on the sarcosine-serine synthesizing system is not duplicated by D-methionine, an active substrate of D-amino acid oxidase. It appears, therefore, that D-alanine is an inhibitor of the serine-synthesizing enzyme.

The experiments with pyruvate show that the serine-synthesizing enzyme and the formaldehyde-pyruvate condensing enzyme are two different enzymes. Just as formaldehyde cannot replace active formaldehyde in serine synthesis, so active formaldehyde cannot replace formaldehyde in the enzymatic synthesis of  $\alpha$ -keto- $\gamma$ -hydroxybutyric acid. Here we have an example of the specific synthetic potential of formaldehyde that was mentioned earlier in this paper. There is, in my opinion, little doubt that many synthetic reactions exist which are specific for either active formaldehyde or for ordinary formaldehyde.

### CYSTEINE AND HOMOCYSTEINE

In the course of our experiments we have had occasion to add L-cysteine to the sarcosine-mitochondrial system. The addition of cysteine eliminated formaldehyde accumulation, although serine synthesis was unaffected (Table 20). This additional differentiation between formaldehyde and active formaldehyde led to a study of the reaction of formaldehyde and cysteine which is still in progress.

When cysteine was added to washed mitochondria suspended in our intracellular buffer (21), containing magnesium but no calcium, at pH 7.8, there was an appreciable induction period during which time there was little or no oxygen uptake. Once oxygen uptake began it followed a sigmoid curve which leveled off when one  $\mu$  atom O had been taken up per  $\mu M$ . of cysteine, a quantity twice



TABLE 20

ACTION OF CYSTEINE ON FORMALDEHYDE AND SERINE ACCUMULATION  
FROM SARCOSINE INCUBATED WITH MITOCHONDRIA

	O	$\mu$ Moles CH <sub>2</sub> O	Serine
10 $\mu$ M. Sarcosine	10.1	3.1	5.6
10 $\mu$ M. Sarcosine + 16 $\mu$ M. Cysteine	20.1	0.6	5.3
16 $\mu$ M. Cysteine	9.4	0	0

that required for the oxidation of cysteine to cystine. This oxidation of cysteine by mitochondria appears to be enzymatic, since with heat-denatured mitochondria, the oxygen uptake is even less than the low level obtained with cysteine in buffer alone.

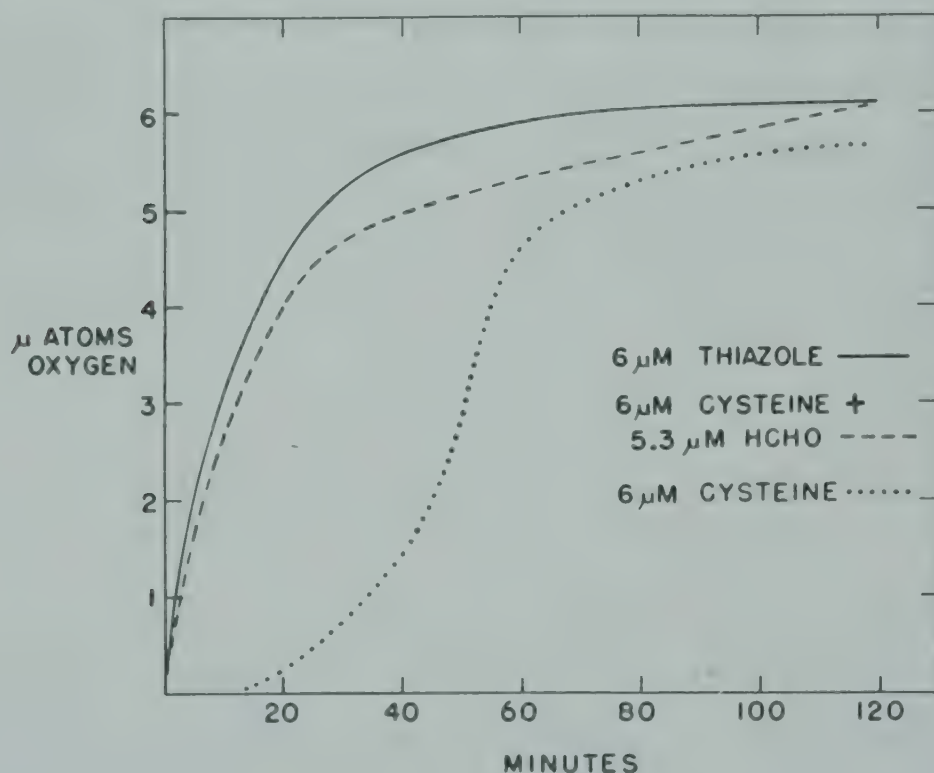


Fig. 10. The uptake of oxygen in a sarcosine mitochondrial system, as affected by cysteine and by cysteine plus formaldehyde.

When, in addition to cysteine, 5.3  $\mu$ M. of formaldehyde were added to the mitochondria from the opposite side-arm of the Warburg flask, the induction period was eliminated and there was an immediate and vigorous uptake of oxygen. As shown in Fig. 10, the oxygen uptake with cysteine plus formaldehyde also ceased when one  $\mu$  atom O had been consumed per  $\mu$ M. cysteine. However, despite

the identity of the oxygen consumed, formaldehyde disappeared completely, as indicated by the chromotropic acid test. *Moreover, it was not regenerated by oxidizing the TCA-soluble fraction with periodate.*

Some observations concerning the reaction between formaldehyde and sulfhydryl compounds are pertinent at this point. When cysteine and formaldehyde are mixed in buffer and shaken for two hours, the slow oxygen uptake normally observed with cysteine alone is eliminated, and the formaldehyde disappears completely. However, the formaldehyde may be regenerated by periodate oxidation at pH 2.4. Similar results are obtained when homocysteine and formaldehyde are shaken in buffer. Glutathione, on the other hand, removes only a small quantity of formaldehyde from solution, as judged by the direct chromotropic acid reaction, while thiourea removes none. These observations suggested that the complete disappearance of free and periodate-liberated formaldehyde from mitochondria in the presence of cysteine was enzymatic in nature.

It is known from the experiments of Schubert (60) and of Ratner and Clarke (61) that formaldehyde and cysteine react rapidly, particularly at pH 5 and above, to form thiazolidine carboxylic acid and it appeared that this compound might be an intermediate in the enzymatic disposal of formaldehyde described above. Consequently thiazolidine carboxylic acid was synthesized from formaldehyde and L-cysteine. This compound gave a negative chromotropic acid test for formaldehyde. However, upon oxidation with periodate at pH 2.4, formaldehyde was formed quantitatively (49). Incubation of the thiazolidine with washed mitochondria resulted in an immediate oxygen uptake which proceeded at a faster rate than was observed when cysteine and formaldehyde were added consecutively to the same preparation (see Fig. 10). At the end of the reaction no formaldehyde was obtained either before or after periodate oxidation of the TCA filtrate. These results demonstrate the existence in mitochondria of a thiazolidine carboxylic acid oxidase. When formaldehyde in excess of cysteine was added to the mitochondria, only that portion of the formaldehyde disappears that was equivalent to the cysteine. Thus thiazolidine carboxylic acid appears to be a



rapidly formed metabolite whose oxidation results in the disposal of both cysteine and formaldehyde. Whether cysteine administration will be of benefit in methanol poisoning remains to be seen.

It was found in parallel experiments that homocysteine, following an induction period, was oxidized by mitochondria at a rate essentially identical with that obtained for cysteine. Indeed, the curve shown in Fig. 10 for cysteine represents DL-homocysteine equally well. However, when formaldehyde was added the oxygen uptake was completely abolished and a compound was formed which exhibited an  $R_f$  of approximately 0.85 in the water-phenol system. This compound appears to be identical with a compound we have synthesized from homocysteine and formaldehyde. It is nitroprusside-negative and it does not give a direct chromotropic acid test for formaldehyde. However, formaldehyde is liberated by periodate oxidation. When homocysteine was added to sarcosine incubated with mitochondria, formaldehyde accumulation was reduced, and paper chromatography revealed a substance exhibiting the  $R_f$  of the homocysteine-formaldehyde addition compound. Serine synthesis did not appear to be substantially decreased.

The difference in behavior of formaldehyde and homocysteine, on the one hand, and formaldehyde and cysteine, on the other, is striking, and appears to me to be of prime importance with respect both to the metabolism of one-carbon compounds and S-amino acids. The rapid formation of thiazolidine carboxylic acid and its oxidation by mitochondria can prevent the accumulation of S-methylolcysteine and the possible synthesis of S-methyl cysteine. Furthermore, it provides a pathway for the rapid metabolism of both cysteine and formaldehyde.<sup>10</sup> On the other hand, the stability in mitochondria of the homocysteine-formaldehyde compound provides a pathway for the synthesis of methionine from homocysteine and formaldehyde. Alternatively, it may be an intermediate in the oxidation of the methyl group of methionine to  $\text{CO}_2$  by tissue slices (see Table 1) without the formation of free formaldehyde (25). In any event, there can be little doubt that the ability of cysteine and homocysteine to react

<sup>10</sup> Formate has recently been found by Dr. Joseph Harris and myself to be a product of the mitochondrial oxidation of thiazolidine carboxylic acid.



with free formaldehyde, but not with the entity we have designated as "active formaldehyde," and the subsequent difference in behavior of the two formaldehyde addition compounds in mitochondrial preparations are matters of metabolic significance.

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# THE SUCCINATE-GLYCINE CYCLE

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MANY OVER-ALL aspects of glycine metabolism are now known, and especially the metabolic pattern of the  $\alpha$ -carbon atom of glycine in its utilization for the synthesis of other compounds has been established. The  $\alpha$ -carbon atom of glycine, no longer attached to the carboxyl group, is utilized for the synthesis of porphyrins (1, 14, 16, 35), the ureido groups of purines (9), the  $\beta$ -carbon atom of serine (19, 32) and for methyl groups (2, 30). This metabolic pattern is similar to that of the so-called "C<sub>1</sub>" compounds, with the exception that the latter cannot substitute for glycine in porphyrin synthesis. It would seem therefore that these apparently unrelated compounds (porphyrins, purines, serine, and methyl groups) have one common feature, namely, the participation of the  $\alpha$ -carbon atom of glycine for their synthesis. The problem of glycine metabolism may then be confined, in this discussion, to the mechanisms by which its  $\alpha$ -carbon atom is utilized for the different compounds mentioned, to the mechanisms by which its  $\alpha$ -carbon atom is detached from its carboxyl group, and also to the mechanism by which glycine carbon atoms are oxidized to carbon dioxide. If we assume, for the purpose of discussion, that in the pathway of glycine metabolism intermediates are produced which are utilized both for porphyrin synthesis and for the synthesis of the other compounds, then postulated mechanisms of glycine metabolism which do not produce intermediates which are utilized for porphyrin synthesis must be looked upon with a certain amount of caution. One postulated pathway of glycine metabolism (glycine  $\rightarrow$  glyoxylate  $\rightarrow$  formate  $\rightarrow$  CO<sub>2</sub>) (29) does not fill the above metabolic requirement, since none of the intermediates (see below) is utilized for porphyrin synthesis.



A pathway for glycine metabolism suggested itself from a study of the mechanism of porphyrin synthesis. In this pathway, called the succinate-glycine cycle (Fig. 1) (20, 27), it is postulated that "active" succinate condenses on the  $\alpha$ -carbon atom of glycine to give rise to  $\alpha$ -amino- $\beta$ -keto adipic acid. This  $\beta$ -keto acid decarboxy-

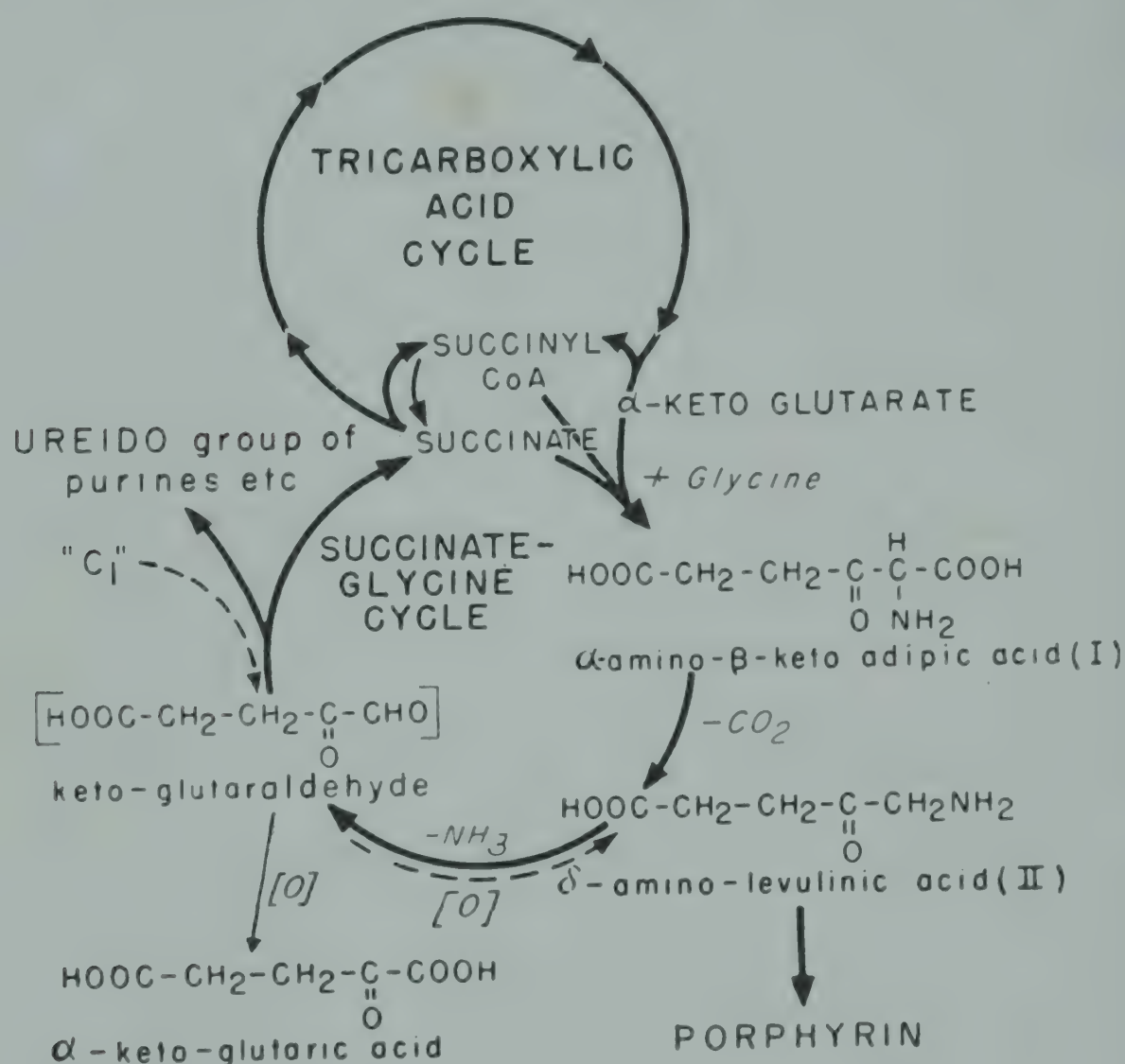
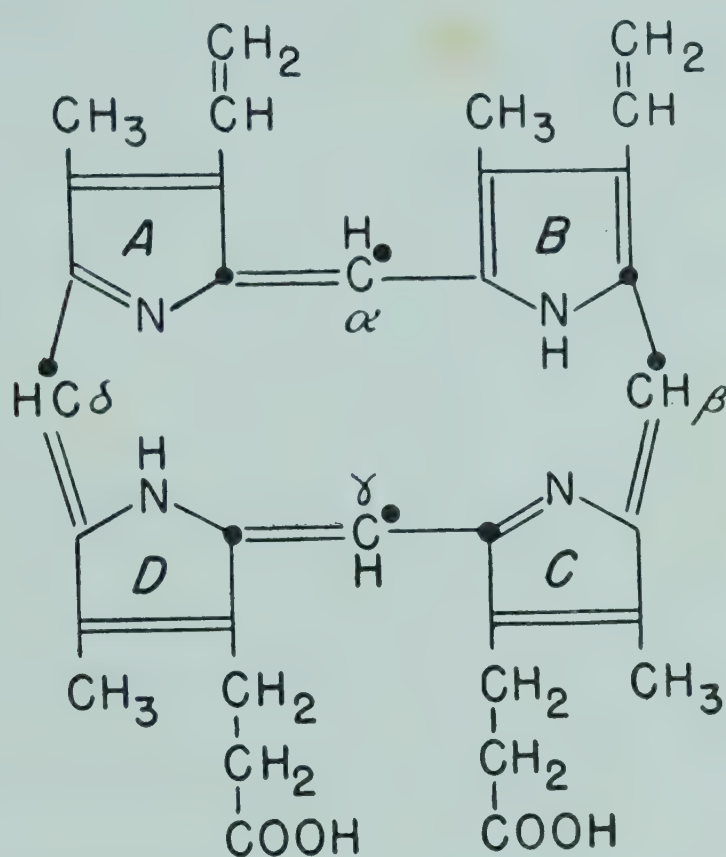


FIG. 1. The succinate-glycine cycle: a pathway for glycine metabolism.

lates to give rise to  $\delta$ -aminolevulinic acid, which can be utilized for porphyrin synthesis or be further catabolized in such a manner that its  $\delta$ -carbon atom (originally the  $\alpha$ -carbon atom of glycine) is utilized for the synthesis of the ureido groups of purines, the  $\beta$ -carbon atom of serine, and for methyl groups, while the remaining four-carbon atom residue is reconverted to succinate. Since this succinate-glycine cycle was arrived at from our study of porphyrin synthesis, it may then be worthwhile to consider the pertinent facts of porphyrin synthesis which led to the elaboration of this cycle, and the findings

which lend their support and broaden the implications of the postulated series of reactions.

It has been previously demonstrated that although the four nitrogen atoms of protoporphyrin are derived from glycine (13, 25, 26, 33, 34), eight carbon atoms of the porphyrin molecule are derived from the  $\alpha$ -carbon atom of glycine (14, 16, 35); four of



## PROTOPORPHYRIN 9

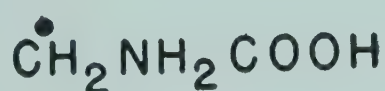


FIG. 2. Positions in protoporphyrin derived from the  $\alpha$ -carbon atom of glycine.

these carbon atoms are still attached to the nitrogen atom (35), and four are not (14, 35). The carbon atoms of protoporphyrin derived from the  $\alpha$ -carbon atom of glycine are shown in Fig. 2. Furthermore, it has been found that the carboxyl group of glycine is not utilized for any of the carbon atoms of the porphyrin (8, 16). It has also been demonstrated that a succinyl intermediate, arising from the tricarboxylic acid cycle, is the source of the remaining twenty-six carbon atoms of protoporphyrin (22, 28). The relation-



ship of the tricarboxylic acid cycle to porphyrin formation was formulated previously as shown in Fig. 3. This relationship was further documented by studying the synthesis of protoporphyrin from  $\alpha$ -ketoglutarate-5- $C^{14}$ ,  $\alpha$ -ketoglutarate-1,2- $C^{14}$  and citrate-1,5- $C^{14}$ . In each case the predicted carbon atoms of the porphyrin were labeled from the  $C^{14}$ -labeled substrate (36). The experiments in these studies were carried out by incubating either whole (23, 24) or hemolyzed red blood cells (12, 21, 22) of a duck with the  $C^{14}$ -

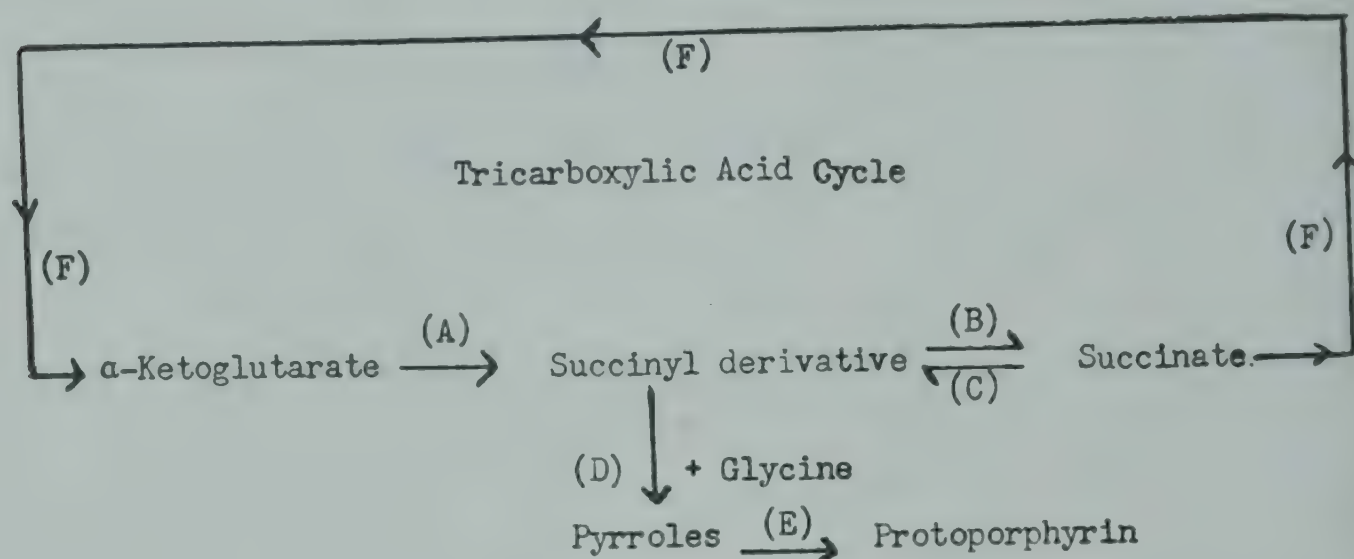


FIG. 3. The relationship of the citric acid cycle to protoporphyrin formation.

labeled compounds, and subsequently chemically degrading the isolated hemin. The degradation was done in such a manner that individual carbon atoms from a particular position in the porphyrin could be isolated (28, 35). The carbon atoms arising from succinate and glycine are shown in Fig. 4. It would appear merely from the  $C^{14}$ -labeling pattern obtained that each pyrrole unit is made up of two moles of succinate and one mole of glycine (22). Since the methene bridges also arise from the  $\alpha$ -carbon atom of glycine, the synthesis of the porphyrin molecule requires eight moles of both succinate and glycine.

It then became of interest to find the chemical mechanism by which the succinate and glycine combine to form the pyrrole unit of the porphyrin. Our studies on the utilization of the  $\alpha$ -carbon of glycine for porphyrin formation have led us to the following mechanism. It was difficult for some time to understand the distri-

bution of the  $\alpha$ -carbon atom of glycine in the porphyrin molecule (four in the pyrrole rings and the four methene bridge carbon atoms, Fig. 2). A possible explanation for the found distribution of the

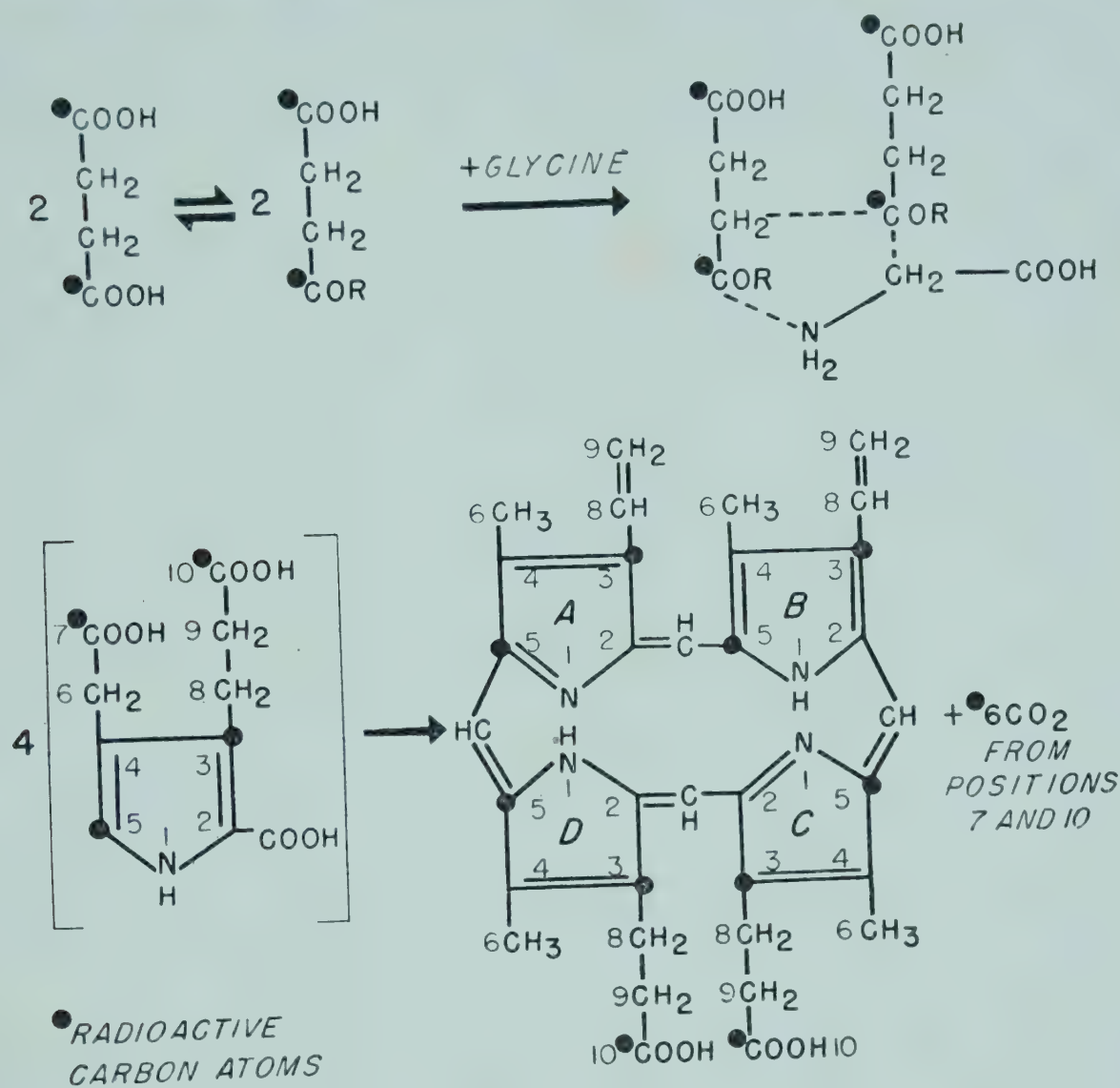


FIG. 4. The labeling pattern in protoporphyrin, showing positions of succinate and the  $\alpha$ -carbon atom of glycine.

$\alpha$ -carbon atom of glycine was that the glycine was utilized for porphyrin synthesis via two pathways.

"x"  $\rightarrow$  Pyrrole ring carbon atom  
 Glycine  $\nearrow$   
 "y"  $\rightarrow$  Methene bridge carbon atom

Since the metabolic pattern of the  $\alpha$ -carbon atom of glycine was similar to that of the " $C_1$ " compounds, and since the methene bridge carbon atoms are no longer attached to the nitrogen atom, it would appear that a " $C_1$ " compound might be substituted for com-



pound "y." Studies were undertaken to test this point. In no case could  $\text{CH}_3\text{OH}$ ,  $\text{H}_2\text{CO}$ ,  $\text{HCOOH}$ ,  $\text{CH}_3\text{NH}_2$ ,  $\text{CO}_2$  or  $\text{CHO}-\text{COOH}$  substitute for glycine (17). These negative findings, coupled with our finding that the  $\alpha$ -carbon atom of glycine was always *equally* utilized for both the pyrrole-ring carbon atom and the methene-bridge carbon atom led us to conclude that *the same derivative of glycine was utilized for both of these positions*.

These findings limit the number of possible ways in which the succinate and glycine combine to form a pyrrole unit. The mechanism of condensation of succinate and glycine becomes a bit more obvious if one considers that the product of this condensation should not only supply a reasonable mechanism of pyrrole formation, but should also suggest a reasonable mechanism by which the  $\alpha$ -carbon atom of glycine is detached from its carboxyl group and should explain the distribution of the  $\alpha$ -carbon atom of glycine in the porphyrin molecule. The condensation of succinate on the  $\alpha$ -carbon atom of glycine to form  $\alpha$ -amino- $\beta$ -ketoadipic acid would appear to agree with the above findings. The compound formed, being a  $\beta$ -keto acid, could then readily be decarboxylated, and thus provide a mechanism by which the  $\alpha$ -carbon atom of glycine becomes detached from its carboxyl group. It would also resolve the apparent paradox that while only the  $\alpha$ -carbon atom of glycine is utilized for porphyrin synthesis, yet no " $\text{C}_1$ " compound can act as a substitute. The product of the decarboxylation would then be  $\delta$ -aminolevulinic acid. Condensation of two moles of the latter, by a Knorr type of condensation (Fig. 5), would readily give rise to a pyrrole in which the  $\alpha$ -carbon atom of glycine would be distributed in the positions previously observed. To test this hypothesis  $\delta$ -aminolevulinic acid was synthesized in three independent ways (17). This was done to insure us of the correctness of the structure. The compound synthesized had the correct analysis and took up one mole of periodate. From the periodate reaction the expected products, formaldehyde and succinic acid, were isolated in excellent yields. Prior to our synthesis of  $\delta$ -aminolevulinic acid, Wynn and Corwin (37) had prepared this compound by another series of reactions.

In the initial experiments, unlabeled  $\delta$ -aminolevulinic acid was added to duck red blood cell hemolysates along with either  $C^{14}$ -labeled glycine or  $C^{14}$ -labeled succinate. The radioactivities of the hemin samples isolated in these experiments were compared with those obtained from controls in which the unlabeled  $\delta$ -aminolevulinic acid was omitted. The rationale for these dilution-type of experi-

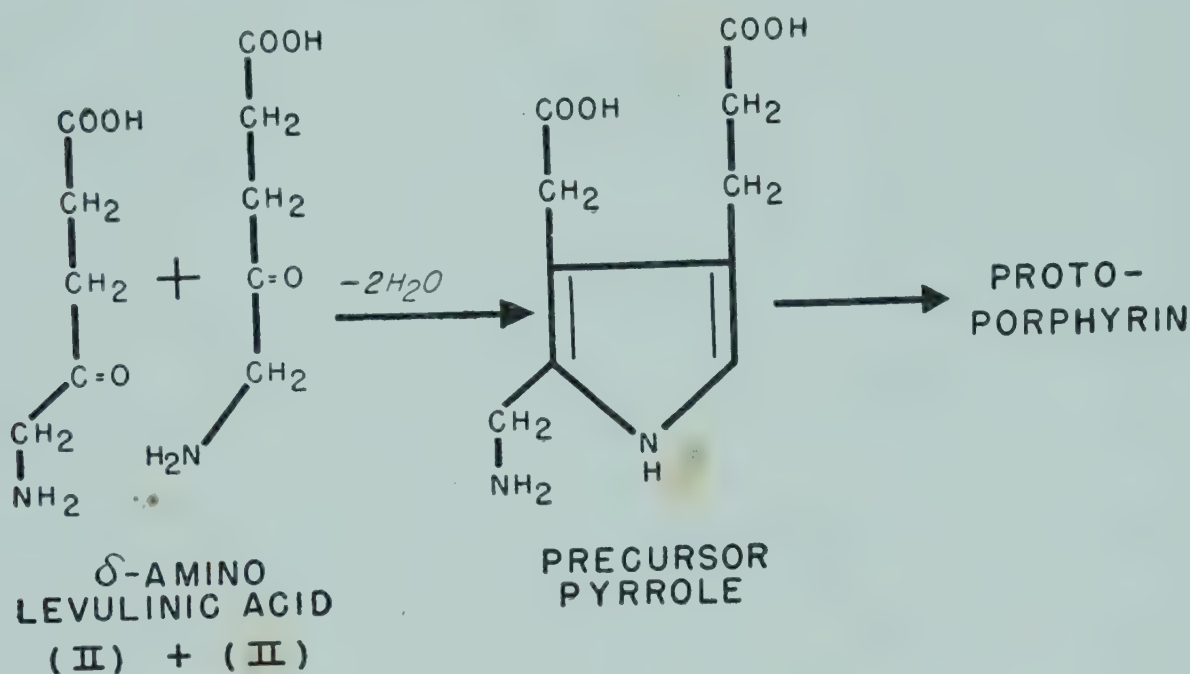


FIG. 5. The formation of the precursor pyrrole for porphyrins from two moles of  $\delta$ -aminolevulinic acid.

ments is as follows: if  $\delta$ -aminolevulinic acid is an intermediate formed from the condensation of glycine and succinate, any labeled  $\delta$ -aminolevulinic acid formed from these labeled substrates will be diluted by the added unlabeled compound, and consequently this should be reflected in the lowered radioactivity of the hemin samples synthesized in the presence of unlabeled  $\delta$ -aminolevulinic acid. It can be seen from Table 1 that the hemin samples made in the presence of unlabeled  $\delta$ -aminolevulinic acid contained less  $C^{14}$  than those of the controls made either from  $C^{14}$ -labeled glycine or succinate (27). These results, which are in full agreement with the hypothesis, can also be explained, however, by the possibility that  $\delta$ -aminolevulinic acid is acting not as a diluent but as an inhibitor of heme synthesis. To rule out the latter possibility, the  $\delta$ -aminolevulinic acid added in experiment 2 (Table 1) was labeled with  $N^{15}$ . It can



TABLE 1

COMPARISON OF  $C^{14}$  ACTIVITIES OF HEMIN SYNTHESIZED FROM GLYCINE-2- $C^{14}$  AND SUCCINIC ACID-2- $C^{14}$  IN PRESENCE AND ABSENCE OF NON-RADIOACTIVE  $\delta$ -AMINOLEVULINIC ACID

Exp. No.	Labeled Substrate	Other Additions	Isotope Conc. in Hemin	
			$C^{14}$	$N^{15}$
1	$C^{14}$ -Succinate (0.1 mM.)	—	c. p. m. 330	atom per cent excess
	$C^{14}$ -Succinate (0.1 mM.)	$\delta$ -aminolevulinic acid 0.1 mM.	90	
2	$C^{14}$ -Glycine (0.05 mM.)	—	230	
	$C^{14}$ -Glycine (0.05 mM.)	$N^{15}$ -labeled $\delta$ -aminolevulinic acid 0.05 mM.	48	0.21
	$N^{15}$ -Glycine (0.33 mM.)			0.05

be seen that whereas the incorporation of  $C^{14}$  from the glycine was lowered, there was a comparatively large incorporation of  $N^{15}$  into the porphyrin, thus demonstrating that the lowered  $C^{14}$  activity of the hemin sample was due to dilution rather than inhibition. Further proof that  $\delta$ -aminolevulinic acid is a result of the condensation of glycine and succinate was obtained by incubating red blood cell hemolysates with glycine-2- $C^{14}$  and unlabeled  $\delta$ -aminolevulinic acid, and subsequently isolating the  $\delta$ -carbon atom. In such an experiment it was found that the formaldehyde liberated upon periodate oxidation of a crude fraction containing  $\delta$ -aminolevulinic acid was highly radioactive.

It now remained to establish more directly the utilization of  $\delta$ -aminolevulinic acid for porphyrin formation. The compound was synthesized with  $C^{14}$  in its  $\delta$ -carbon atom, and its utilization was compared with that of glycine. It can be seen from Table 2 that the hemin synthesized from an equimolar amount of  $\delta$ -aminolevulinic acid was about 50 times more radioactive than hemin synthesized from glycine. More rigorous proof that  $\delta$ -aminolevulinic acid is indeed the precursor for porphyrin synthesis was obtained by degrading a hemin sample synthesized from  $\delta$ -aminolevulinic acid-5-

TABLE 2

COMPARISON OF  $C^{14}$  ACTIVITIES IN HEMIN SYNTHESIZED FROM GLYCINE-2- $C^{14}$  (0.05 MC./MM.) AND  $\delta$ -AMINOLEVULINIC ACID-5- $C^{14}$  (0.05 MC./MM.)

Labeled Substrate	Non-radioactive Additions	$C^{14}$ Activity in Hemin
$C^{14}$ -Glycine (0.05 mM.)	Succinate (0.1 mM.)	c. p. m. 480
$C^{14}$ - $\delta$ -aminolevulinic acid (0.05 mM.)	—	23,000
$C^{14}$ - $\delta$ -aminolevulinic acid (0.05 mM.)	Glycine (0.33 mM.) Succinate (0.1 mM.)	21,000

$C^{14}$ . The  $\delta$ -carbon atom of the latter compound should label the same carbon atoms of protoporphyrin as those which we have previously found to arise from the  $\alpha$ -carbon atom of glycine, since according to the hypothesis the latter carbon atom is the biological source of the  $\delta$ -carbon atom of  $\delta$ -aminolevulinic acid.

TABLE 3

DISTRIBUTION OF  $C^{14}$  ACTIVITY IN PROTOPORPHYRIN SYNTHESIZED FROM  $\delta$ -AMINOLEVULINIC ACID-5- $C^{14}$  AND FROM GLYCINE-2- $C^{14}$

Fragments of porphyrin	Molar activity (%) in fragments of porphyrin synthesized from	
	$\delta$ -Aminolevulinic acid-5- $C^{14}$ , %	Glycine-2- $C^{14}$ , %
Protoporphyrin	100	100
Pyrrole rings A + B (methylethylmaleimide)	24.5	24.6
Pyrrole rings C + D (Hematinic acid)	25.2	25.3
Pyrrole rings A + B + C + D	49.7	49.9
Methene bridge carbon atoms	50.3	50.1

It can be seen from Table 3 that the same  $C^{14}$  distribution pattern was found in protoporphyrin synthesized from  $\delta$ -aminolevulinic acid-5- $C^{14}$  as from glycine-2- $C^{14}$ ; 50 per cent of the  $C^{14}$  activity resides in the pyrrole rings and 50 per cent in the methene bridge carbon atoms (see Fig. 2) (20). The utilization of  $\delta$ -aminolevulinic



acid for porphyrin synthesis has been subsequently confirmed in two instances (4, 15). Furthermore, it may be well to point out that the theoretical formulation of the structure of the precursor pyrrole (27) is the same structure which was determined for porphobilinogen (3, 10, 31), a compound excreted in the urine of patients with acute porphyria. The utilization of  $\delta$ -aminolevulinic acid for porphyrin formation adds to the certainty that porphobilinogen (5) is an intermediate in porphyrin synthesis. These findings make  $\alpha$ -amino- $\beta$ -ketoadipic acid an obligatory intermediate.

The condensation of "active" succinate and glycine to form  $\delta$ -aminolevulinic acid subsequently, thus far appears to require the partially intact structure of the red blood cell. It has been found that whereas  $\delta$ -aminolevulinic acid can be converted to protoporphyrin in either an homogenized preparation or a cell-free extract, or in a lyophilized preparation of a cell-free extract (20), the conversion of succinate and glycine to porphyrin only takes place with intact cells or with those cells which have been hemolyzed with water. Homogenized preparations obtained in a blender are no longer capable of synthesizing protoporphyrin from succinate and glycine (Table 4) (20). It would appear that on homogenization the functional activity of only those enzymes of the system that are involved in the condensation of succinate and glycine is lost.

If the proposed succinate-glycine cycle is a pathway of glycine metabolism embracing the reactions known for the  $\alpha$ -carbon atom of glycine, then the  $\delta$ -carbon atom of  $\delta$ -aminolevulinic acid should have the same metabolic spectrum. To test the hypothesis,  $\delta$ -aminolevulinic acid-5- $C^{14}$  was injected into a duck, and hemin, guanine, and adenine were isolated from the red blood cells, serine and the methyl group of methionine were obtained from the plasma proteins, and uric acid was isolated from the excreta.

It was found that the ureido groups of the purines (18), the  $\beta$ -carbon atom of serine (7), and the methyl group of methionine (6) contained  $C^{14}$ . These experimental results, which support the existence of the succinate-glycine cycle, are only to be considered as preliminary in nature, since quantitative aspects have not yet been

TABLE 4

COMPARISON OF  $C^{14}$  ACTIVITIES OF HEMIN SYNTHESIZED FROM  $\delta$ -AMINOLEVULINIC ACID-5- $C^{14}$  (0.05 MC./MM.) AND SUCCINIC ACID-2- $C^{14}$  (0.05 MC./MM.) IN DIFFERENT RED BLOOD CELL PREPARATIONS

Expt.	Substrate, acid	Red cell preparation	$C^{14}$ activity in hemin sample, c. p. m.
1	Succinic (0.05 mM.) <sup>a</sup>	Hemolyzed	390
	Succinic (0.05 mM.) <sup>a</sup>	Homogenized	7
2	$\delta$ -Aminolevulinic (0.013 mM.)	Hemolyzed	4300
	$\delta$ -Aminolevulinic (0.013 mM.)	Homogenized	4500
3	$\delta$ -Aminolevulinic (0.009 mM.)	Homogenized	2200
	$\delta$ -Aminolevulinic (0.009 mM.)	Supernatant ( $12 \times 10^8$ g)	1600
	$\delta$ -Aminolevulinic (0.009 mM.)	Supernatant ( $47 \times 10^8$ g)	1600
	$\delta$ -Aminolevulinic (0.009 mM.)	Supernatant ( $100 \times 10^8$ g)	1500
4	$\delta$ -Aminolevulinic (0.009 mM.)	Supernatant ( $12 \times 10^8$ g)	2000
	$\delta$ -Aminolevulinic (0.009 mM.)	Lyophilized prepn.	1500

<sup>a</sup> Plus 0.33 mM. of non-radioactive glycine.

completely explored. Quantitative evaluation is difficult in whole animals, since cells are not very permeable to  $\delta$ -aminolevulinic acid and the compound is very unstable as the free amino acid. In an experiment in the bird the  $C^{14}$  activity of both the hemin and the ureido groups of the purines synthesized from  $\delta$ -aminolevulinic acid-5- $C^{14}$  was 2.5 times greater than in those samples synthesized in a comparable experiment in which glycine-2- $C^{14}$  was the substrate. This may be a minimal difference if one considers the experiments in vitro (see Table 2) in which the  $C^{14}$  activity of the hemin synthesized from  $\delta$ -aminolevulinic acid was about 50 times greater than that synthesized from glycine. However, it would appear from the above results that the  $\delta$ -carbon atom of  $\delta$ -aminolevulinic acid has the same metabolic pattern as that of the  $\alpha$ -carbon atom of



glycine and likewise as that of the "C<sub>1</sub>" compounds beyond the porphyrin synthesis. Further support for this conclusion is our finding that while both glycine-2-C<sup>14</sup> and  $\delta$ -aminolevulinic acid-5-C<sup>14</sup> gave rise to radioactive formate in the urine of a rat, the formate arising from  $\delta$ -aminolevulinic acid was about 3 times more radioactive than that made from the  $\alpha$ -carbon atom of glycine (7).

The cycle also provides a mechanism by which the carbon atoms of glycine can be oxidized to carbon dioxide. The formation of  $\alpha$ -amino- $\beta$ -ketoadipic acid and subsequent decarboxylation of this  $\beta$ -keto acid would yield one mole of carbon dioxide. This mole of carbon dioxide was originally the carboxyl group of glycine. If the postulated ketoglutaraldehyde or some other derivative can be converted to  $\alpha$ -ketoglutaric acid, a pathway is provided for the conversion of the  $\alpha$ -carbon atom of glycine to carbon dioxide, for the  $\alpha$ -carboxyl group of the ketoglutarate was originally the  $\alpha$ -carbon atom of glycine. In this mechanism, it will be noted, formate is not an obligatory intermediate for the conversion of the  $\alpha$ -carbon atom of glycine to carbon dioxide.  $\alpha$ -Ketoglutaric acid isolated from a cell-free extract of duck red blood cells which were incubated with  $\delta$ -aminolevulinic acid-5-C<sup>14</sup> contained radioactivity. Further, the carbon-6 atom of uric acid which arises from carbon dioxide was found to be radioactive in an experiment in which  $\delta$ -aminolevulinic acid-5-C<sup>14</sup> was injected into a duck and into a pigeon (18). It may be well to point out that in this experiment the C<sup>14</sup> activity of carbon atoms 2 and 6 was approximately the same (18). The significance of this finding is not altogether clear, but it may be suggested that perhaps a derivative formed from  $\delta$ -aminolevulinic acid is the "active" carbon dioxide derivative which is utilized for the carbon-6 atom of uric acid.

Although the evidence for the condensation of succinate and glycine appears to be rather conclusive, it does appear that this reaction may not be unique but rather a prototype of a more general reaction. We observed a few years ago that the utilization of glycine for porphyrin formation is markedly inhibited by the addition of either acetate or pyruvate (11). These compounds appear to



inhibit the condensation of succinate and glycine, since the conversion of  $\delta$ -aminolevulinic acid to porphyrin is not influenced by the addition of these acids. It would appear that "active" acetate may compete with succinate for the glycine, since the inhibition can be overcome by the addition of other members of the citric acid cycle. If acetate condenses with glycine the product would be  $\alpha$ -aminoacetoacetic acid. Upon decarboxylation this  $\beta$ -keto acid would yield aminoacetone, an analogue of  $\delta$ -aminolevulinic acid. We have carried out some preliminary studies with aminoacetone labeled with  $C^{14}$  in the carbon atom bearing the amino group. We have found that this radioactive carbon atom is utilized for the ureido groups of uric acid, that it can be converted both to formate and carbon dioxide the radioactivity of which is several times greater than that produced from glycine-2- $C^{14}$  (7). It still remains to be proved that aminoacetone can be synthesized by the cell.

The condensation of glycine with either "active" succinate or "active" acetate provides a pathway whereby glycine can be oxidized to carbon dioxide and the intermediates produced in the cycle drawn off for the synthesis of other compounds. This is similar to the citric acid cycle, in which another two-carbon compound is oxidized to carbon dioxide and intermediates are produced which can be drawn off for synthesis. In the succinate-glycine cycle, succinate is the catalyst instead of oxaloacetate.

In order to unify the reactions of glycine, it may be suggested that in reactions in which the whole molecule of glycine is involved  $\alpha$ -amino- $\beta$ -ketoadipic acid may play a role. The formation of serine, for example, may take place by the reaction between an "active  $C_1$ " compound and succino-glycine ( $\alpha$ -amino- $\beta$ -ketoadipic acid) to yield serine and succinate.

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# THE FORMATION OF GLYCINE FROM RIBOSE-5-PHOSPHATE

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GLYOXYLIC AND GLYCOLIC acids have been shown to be effective precursors of the carbon chain of glycine in the rat, and the conversion of glycolaldehyde to glycine has also been indicated (1, 2, 3). Since recent work has strongly suggested the participation of an active form of glycolaldehyde in carbohydrate metabolism (4, 4a), a possible link between the sugars and glycine has become apparent. Furthermore, photosynthetic studies have shown that glycine and glycolic acids are formed in the early phases of photosynthesis after the initial appearance of the sugar phosphates (5).

We have been investigating a soluble extract from spinach leaves which fixes  $\text{CO}_2$  into phosphoglyceric acid in the presence of ribose-5-phosphate. If 1- $\text{C}^{14}$ -ribose-5-phosphate is used as a precursor in this system, an appreciable amount of radioactivity is found in the glycine isolated from the incubation mixture. This is shown in Table 1. Glycine was isolated from the incubation mixture by removal of the barium insoluble salts, addition of carrier glycine to

TABLE 1  
THE FORMATION OF GLYCINE FROM 1- $\text{C}^{14}$ -RIBOSE-5-PHOSPHATE

Amino acid isolated *	Total counts fixed as c.p.m.	Percentage of total counts fixed	
		Methylene carbon atom	Carboxyl carbon atom
Glycine	$1.94 \times 10^4$	80	16
Serine	$< 100$		
Histidine <sup>+</sup>	$< 100$		
Threonin, + aspartic acid, + glutamic acid	$5 \times 10^2$		

\* The incubation mixture contained 1.0 ml. of spinach extract, 1  $\mu\text{M}$ . of DPN, 5  $\mu\text{M}$ . of ATP, 400 units of glucose dehydrogenase, 200  $\mu\text{M}$ . of glucose, 50  $\mu\text{M}$ .  $\text{K}_2\text{CO}_3$ , 100  $\mu\text{M}$ . of 1- $\text{C}^{14}$ -ribose-5- $\text{PO}_4$  containing  $1.47 \times 10^6$  c.p.m. in a total volume of 2.3 ml. The incubation was at 25° C. for one hour under 100%  $\text{CO}_2$ .

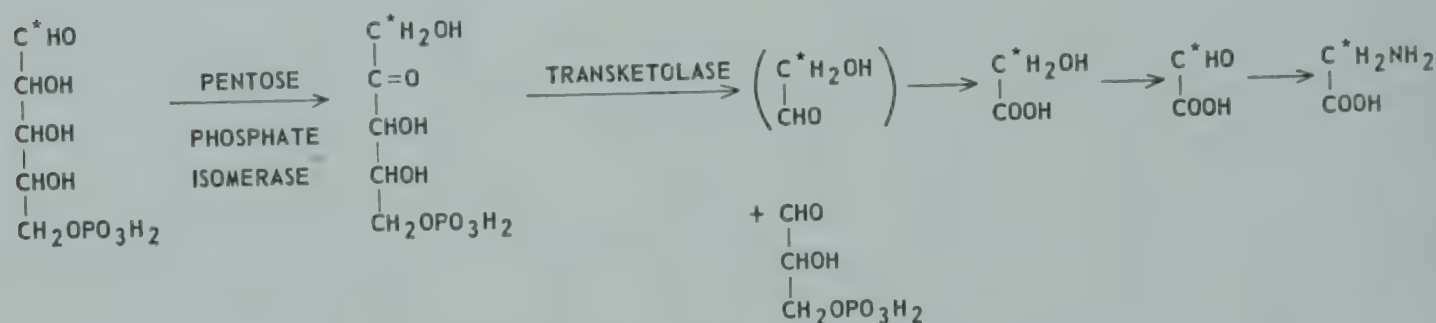
<sup>+</sup> Isolated by Dr. Bruce Ames.

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the supernatant fluid, and chromatography of the mixture on Dowex-50 (6). The identity of the glycine so isolated was established by paper chromatography, using two solvent systems. Chromatography of the N-2,4-dinitrophenyl derivative showed it to have the same  $R_f$  as an authentic sample of N-2,4-dinitrophenylglycine. After degradation with ninhydrin, almost all of the radioactivity of the glycine could be recovered in the HCHO and CO<sub>2</sub> produced. As shown in Table 1, the other amino acids isolated from the incubation mixture in a similar manner to that of the glycine isolation showed low levels of radioactivity.

Since the spinach preparations used contained pentose phosphate isomerase and transketolase, the incorporation of carbon-1 of ribose into the methylene carbon atom of glycine can be explained by the following sequence of reactions:



The minor incorporation of C<sup>14</sup> from carbon-1 of ribose into the carboxyl carbon atom of glycine may take place by reactions which would distribute the C<sup>14</sup> into other carbon atoms of the sugars. Thus, it has been found that 1,3-C<sup>14</sup> hexose phosphate is formed from 1-C<sup>14</sup>-ribose-5-phosphate in spinach leaf preparations (7).

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# INTERRELATIONSHIPS OF AMINO ACID METABOLISM WITH PURINE BIOSYNTHESIS

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THE ROLE which amino acids play in the synthesis of nucleic acid components has long been a matter of investigation by the biochemist. In connection with the synthesis of the purines Wiener suggested in 1902 (37) that the uric acid molecule was synthesized from tartronic acid and two moles of urea. After some 30 years sufficient experimental evidence was obtained to conclude that this long-standing theory was incorrect, and that urea was not the precursor of the ureide structures of the purine molecule. Investigators turned their attention to dietary amino acids as the donors of the nitrogen atoms of the purine ring. The amino acids which received greatest attention were those which structurally had some similarity to the purine ring, viz., histidine and arginine. These amino acids were tested eventually by a variety of techniques in vivo. The one most frequently employed in earlier studies was to fast an animal until uric acid (or allantoin) excretion reached a constant baseline, and then to feed the amino acid in question and note the effect on purine excretion. The results of these various investigations were in considerable conflict (1, 25). This controversy was finally settled when stable and radioactive isotopes became available for research in intermediary metabolism. Neither Tesar and Rittenberg (34) nor Bloch (4) could find any evidence that the nitrogen atoms of the histidine

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ring nor the guanidyl nitrogen atoms of arginine could be converted into purine compounds. This work substantiated the earlier conclusions of Schoenheimer and his colleagues (2) that purine as well as pyrimidine compounds are rapidly formed from simple nitrogenous units in the body and not from such structures as histidine and arginine. These isotopic experiments were largely responsible for removing the straightjacket which had been imposed on the thinking of investigators in this field.

### ROLE OF AMINO ACIDS IN PURINE SYNTHESIS DE NOVO

A new approach was made to the problem simultaneously by Schuler and Reindel (27) and by Krebs and his coworkers (10, 24). These investigators showed that pigeon liver slices synthesized a purine structure, later identified as hypoxanthine, which could be converted to uric acid by the addition of xanthine oxidase. The pigeon liver is unusual in that it lacks this enzyme and that the product of this biosynthetic reaction is the reduced purine, hypoxanthine. This fortunate situation was responsible for the identification of one intermediate in uric acid formation and indicated that the precursor of the 2 and 8 carbon atoms would probably be carbon compounds of a lower oxidation state than  $\text{CO}_2$ . Other observations by Krebs and his coworkers were that oxaloacetate and glutamine or pyruvate,  $\text{CO}_2$ , and ammonium ions were capable of stimulating hypoxanthine synthesis by this system.

The observations (5, 6, 18, 20, 31) made with the use of isotopically labeled compounds that  $\text{CO}_2$  is the precursor of carbon atom 6 of the purine ring, formate of carbon atoms 2 and 8, and glycine of carbon atoms 4 and 5 as well as nitrogen atom 7 provided a complete knowledge of the precursors of all of the carbon atoms as well as one of the four nitrogen atoms. These experiments thus definitely implicated one amino acid in the purine synthetic reactions. When  $\text{N}^{15}$  of  $\text{NH}_4\text{Cl}$  was fed simultaneously with the above precursors to the intact pigeon it was rapidly incorporated into excretory uric acid. Lagerkvist (22) has recently studied the pattern of isotope distribution in the four nitrogen atoms of uric acid thus synthesized with a



new method recently devised in his laboratory. He has found that nitrogen atoms 3 and 9 are derived to a much larger extent from administered  $\text{N}^{15}\text{H}_4\text{Cl}$  than are atoms 1 and 7. The results with  $\text{NH}_4\text{Cl}$ , however, do not reveal the identity of the immediate precursors of the three remaining nitrogen atoms (1, 3, and 9) since  $\text{NH}_3$  is incorporated into a great many organic forms in the body.

*Stoichiometric reaction of precursors in purine synthesis.*

In search for the immediate precursors of the purines, use was made of the fact that conditions had been sufficiently developed to obtain synthesis of hypoxanthine in pigeon liver extracts from its elementary precursors (29). When bicarbonate ion, formate, and glycine were incubated with pigeon liver extracts in elevated concentrations, these substrates were utilized in the synthesis of hypoxanthine in the molecular ratio of 1:2:1. In fact, the reaction of substrates in this definite ratio was taken as the best evidence that synthesis of purine de novo was occurring in the system. Since the carbon precursors of purine synthesis had been shown to participate in the reaction in a definite stoichiometric manner, it might be expected that such stoichiometry would be found when  $\text{N}^{15}$ -labeled substrates were compared with glycine utilization. In a series of experiments by Drs. John C. Sonne and I. Lin (32), the incorporation of  $\text{C}^{14}$  of glycine-1- $\text{C}^{14}$  into hypoxanthine was compared to  $\text{N}^{15}$  utilized from such compounds as  $\text{N}^{15}\text{H}_4\text{Cl}$ ,  $\text{N}^{15}$ -L-aspartic acid,  $\text{N}^{15}$ -L-glutamic acid and  $\text{N}^{15}$ (amide-labeled)-L-glutamine. As shown in Table 1, all of the organic nitrogen compounds contributed significantly to hypoxanthine synthesis in the pigeon liver extract system, whereas ammonium ions, though readily incorporated in vivo, seem far inferior as a precursor in vitro to any of the above three organic nitrogen compounds tested. Since the technical difficulties encountered in this type of experiment (e. g., determination of the change of atom per cent excess  $\text{N}^{15}$  and the specific radioactivity of the isotopic substrates during the course of the incubation) were considerable, it is felt that the final calculation of the molar ratio of  $\text{N}^{15}$ -aspartate, -glutamate, and -glutamine to  $\text{C}^{14}$ -glycine utilized in purine synthesis represents only an approximation. The nearness



TABLE 1  
REACTION OF NITROGENOUS SUBSTRATES  
IN DEFINITE PROPORTIONS FOR HYPOXANTHINE SYNTHESIS \*

Expt.	N <sup>15</sup> -labeled Substrate	Moles of N <sup>15</sup> utilized for hypoxanthine synthesis per mole of C <sup>14</sup> -labeled glycine
1	NH <sub>4</sub> Cl	0.27
2	Aspartic acid	1.20
3	Glutamic acid	1.20
4	Glutamine (amide N <sup>15</sup> )	1.90
5	Glycine	1.00

\* From Sonne, Lin, and Buchanan (32).

of these ratios to integral numbers, however, suggested that these substrates were indeed contributing specifically to one or more of the three remaining nitrogen atoms of the purine ring not supplied by glycine.

*Methods for the chemical degradation of uric acid.*

The best evidence, however, for the belief that these nitrogen compounds are precursors of specific nitrogen atoms of the purine ring comes from chemical degradation of the uric acid molecule. Methods for the determination of the isotope content of each nitrogen atom in this heterocyclic ring system have been under active investigation in this laboratory. We have succeeded in obtaining an isotopic analysis of each individual nitrogen atom of the four nitrogen atoms of uric acid, using a combination of the three chemical degradation methods outlined in Table 2. The first two of these methods are well-known standard degradative procedures (6, 31), while the alkaline peroxide oxidation method has recently been developed in this laboratory by Dr. H. J. Brandenberger for the sole purpose of distinguishing between nitrogen atoms 1 and 3 of the pyrimidine moiety of uric acid. A preliminary report concerning this new purine degradative method has already been published (8).

The mechanism which has been proposed by Brandenberger for the degradation of uric acid by alkaline peroxide is based on his

TABLE 2  
CHEMICAL DEGRADATIONS OF URIC ACID

Method of Degradation	Overall Reaction	Nitrogen Atoms Separated
1. HCl hydrolysis	Uric Acid $\rightarrow$ Glycine + 3NH <sub>3</sub>	(N <sub>7</sub> ) (N <sub>1</sub> + N <sub>3</sub> + N <sub>9</sub> )
2. Chlorine oxidation	Uric Acid $\rightarrow$ Alloxan + Urea	(N <sub>1</sub> + N <sub>3</sub> ) (N <sub>7</sub> + N <sub>9</sub> )
3. Alkaline peroxide oxidation	Uric Acid $\rightarrow$ Oxonic Acid + Ammonia	(N <sub>1</sub> + N <sub>7</sub> ) ([N <sub>1</sub> + N <sub>3</sub> + N <sub>9</sub> ] + [N <sub>7</sub> + N <sub>3</sub> + N <sub>9</sub> ])

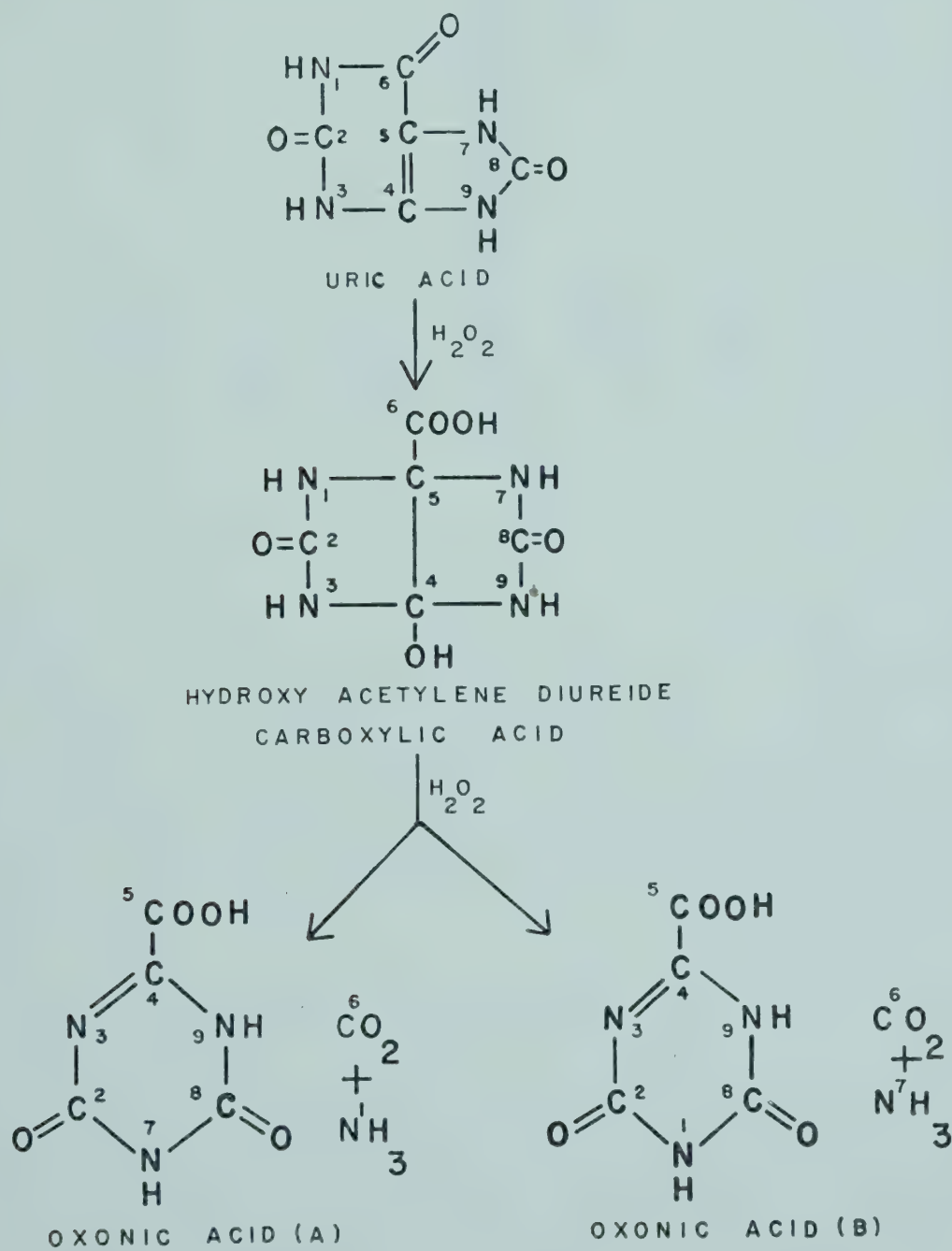


FIG. 1. The oxidation of uric acid by alkaline hydrogen peroxide.

experiments with samples of uric acid labeled in various positions with C<sup>14</sup> and N<sup>15</sup>. The reaction scheme given in Fig. 1 differs con-



siderably from that initially proposed by Venable (35) and by Moore and Thomas (23). It is believed that uric acid is first converted to the symmetrical hydroxyacetylene diureide carboxylic acid, which is further oxidized to yield  $\text{NH}_3$ ,  $\text{CO}_2$ , and oxonic acid. Upon acidification, oxonic acid is converted into allantoxaidin and a second molecule of  $\text{CO}_2$ . Oxonic acid and allantoxaidin have been assigned the structures 2,4-dioxy-5-carboxy-tetrahydro-*s*-triazine and 2,4-dioxy-tetrahydro-*s*-triazine, respectively. The  $\text{NH}_3$  sample is derived equally from  $\text{N}_1$  and  $\text{N}_7$ , while the nitrogen atoms of one isotopic species of oxonic acid represent  $\text{N}_1 + \text{N}_3 + \text{N}_9$  of the original uric acid and the other isotopic species comprises  $\text{N}_7 + \text{N}_3 + \text{N}_9$ . Work in this laboratory by Mr. S. C. Hartman and Dr. J. Fellig (17) independently supports the conclusions originally proposed by Brandenberger.

The experimental observation (see Table 1) that the molar ratio of incorporation of  $\text{N}^{15}$ -glutamine to  $\text{N}^{15}$ -glycine was approximately 2:1 has been extended by use of degradation schemes 1 and 2. It has been found that the amide nitrogen of one of the molecules of glutamine used in hypoxanthine synthesis is the metabolic precursor of nitrogen atom 9 of the ring (32). The amide nitrogen of the second molecule of glutamine is found in the pyrimidine moiety of the base, and work is now in progress to determine the precise distribution of the isotope between nitrogen atoms 1 and 3. By application of all three of the methods given in Table 2, we have been able to demonstrate that the  $\alpha$ -amino nitrogen atom of L-aspartic acid or L-glutamic acid is the source of nitrogen atom 1 of the purine nucleus.

#### *Role of amino acids in the stimulation of purine synthesis.*

In view of the fact that the labeled atoms of the three nitrogenous organic compounds thus far tested appear to be precursors of specific nitrogen atoms of the purine ring in vitro, we have undertaken experiments which attempt to correlate the above isotopic data with the effect which these compounds have on the stimulation of hypoxanthine biosynthesis from glycine-1- $\text{C}^{14}$ .

In order to simplify the enzymatic system wherein the effects of various amino acids on purine biosynthesis could be tested, the proteins of pigeon liver extract were precipitated with ethanol at 45 per cent concentration, and the supernatant fluid containing most

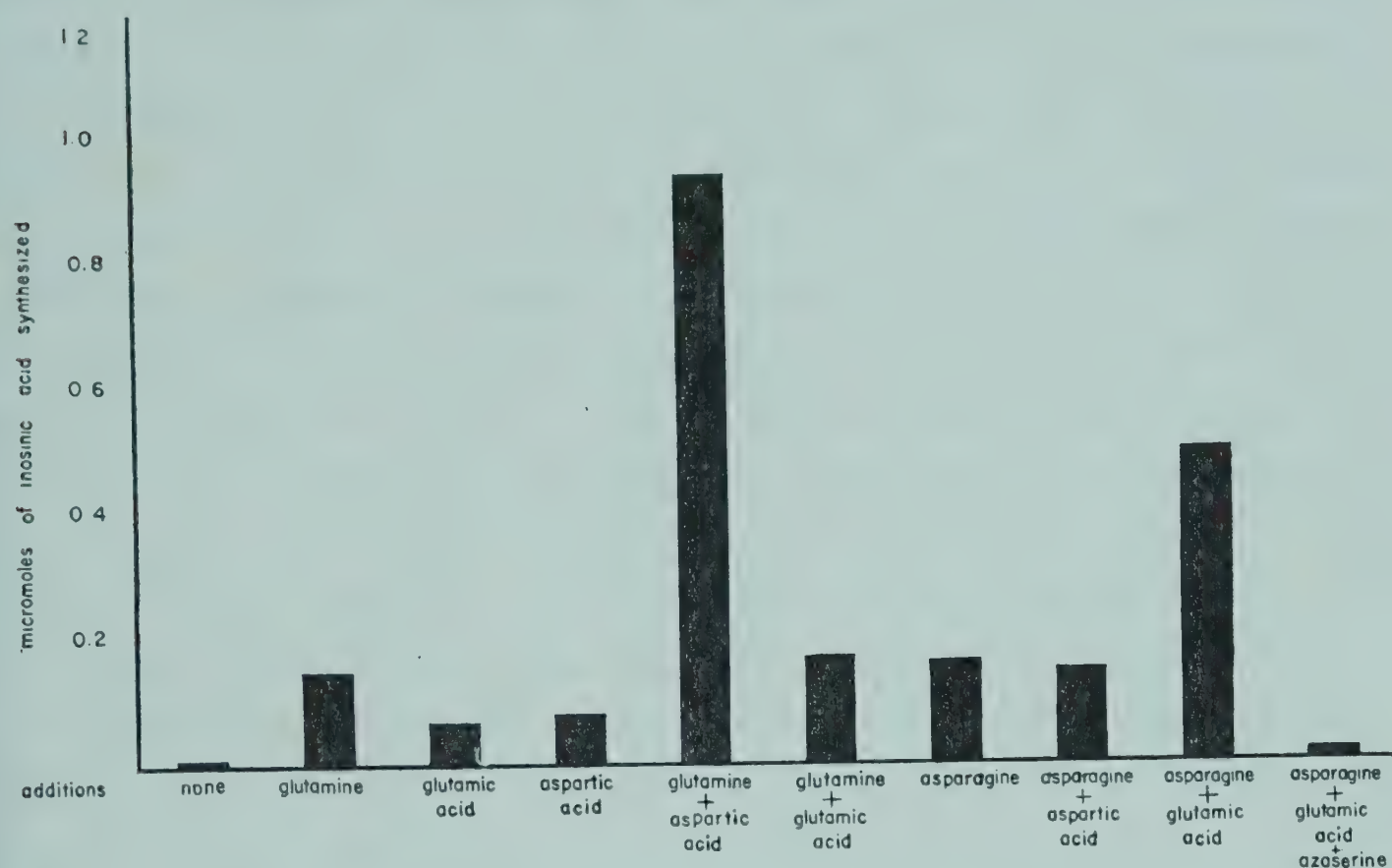


FIG. 2. Effect of amino acids and amides on inosinic acid formation.

The vessels each contained, in a total volume of 1.8 ml., glycine-1-C<sup>14</sup> 14  $\mu$ M., sodium formate 30  $\mu$ M., potassium bicarbonate 60  $\mu$ M., ribose-5-phosphate 20  $\mu$ M., 3-phosphoglyceric acid 15  $\mu$ M., calcium leucovorin 75  $\gamma$ , 0.1 M. phosphate buffer pH 7.4, .5 ml., containing Mg<sup>++</sup> and K<sup>+</sup>, 0.5 ml. of pigeon liver 0-45% ethanol-precipitated enzyme dissolved in a minimal volume of water. L-amino acids or amides incubated separately, as shown, at levels of 15  $\mu$ M. When in combination, each nitrogenous substrate was added at a level of 7.5  $\mu$ M., 4  $\mu$ M. of L-azaserine were added to the final vessel. All vessels were incubated for 2 hours at 38° C., and the reaction was stopped by boiling for 5 minutes. Carrier hypoxanthine (10 mg.) was added, and inosinic acid was hydrolyzed with dilute nitric acid for 30 minutes. Hypoxanthine was isolated as the silver picrate according to the procedure of Schulman, Sonne, and Buchanan (29).

of the organic cofactors and substrates was discarded. The enzymes were redissolved in buffered solution and supplemented, as described in Fig. 2, with substrate quantities of glycine-1-C<sup>14</sup>, formate, bicarbonate, ribose-5-phosphate, 3 phosphoglyceric acid, and with a trace of leucovorin (synthetic citrovorum factor). After 2 hours' incubation of these substrates at 38° C. a small amount of inosinic acid



was synthesized from radioactive glycine. The effect of the addition of various nitrogen-containing compounds to the above enzymatic system resulted in the stimulation of inosinic acid synthesis, as shown in Fig. 2. The inclusion of either L-glutamine or L-asparagine in concentrations of  $15\ \mu\text{M.}/1.8\ \text{ml.}$  of final solution causes an 18-fold increase in inosinic acid formation (from  $0.0085\ \mu\text{M.}$  to  $0.155\ \mu\text{M.}$ ). The  $\alpha$ -amino dicarboxylic acids, L-aspartic acid and L-glutamic acid, likewise have a marked but lesser effect on the rate of reaction when incubated separately. In order to determine whether the dicarboxylic acids are unique in their ability to stimulate synthesis, presumably as precursors of  $\text{N}_1$  of the purine base, a series of other amino acids were tested. In this experiment  $15\ \mu\text{M.}$  each of L-alanine, L-serine, L-threonine, L-phenylalanine, DL-citrulline, L-aspartic acid, and L-glutamic acid were added to individual vessels and compared for their effect on the reaction. Only glutamic and aspartic acids stimulated the reaction markedly. Serine had a small effect, and the remaining amino acids had little or none. It can be concluded from these data that the reaction involving the incorporation of nitrogen atom 1 into the purine nucleus during synthesis *de novo* from its elementary precursors more or less specifically involves the transfer of nitrogen from the  $\alpha$ -amino group of one or both of these dicarboxylic amino acids.

It was of further interest to determine whether an additive or synergistic effect could be obtained by incubating both classes of compounds (i. e., amides and dicarboxylic amino acids) together with the other components of the enzymatic system. Under the conditions of the particular experiment reported in Fig. 2, optimal synthesis of inosinic acid occurred when L-glutamine and L-aspartic acid were incubated together. The vessel contained  $7.5\ \mu\text{M.}$  of each compound or a total of  $15\ \mu\text{M.}$  of added nitrogenous substrates. In this vessel  $0.93\ \mu\text{M.}$  of inosinic acid was formed, whereas only  $0.14\ \mu\text{M.}$  and  $.07\ \mu\text{M.}$  were formed when L-glutamine and L-aspartic acid, respectively, were incubated in separate vessels. It is thus seen that when both compounds are present there is a synergistic effect on the rate of the synthesis, over that obtained when they are incubated



alone. A similar though not as pronounced effect could be obtained when L-asparagine and L-glutamic acid were incubated together.

Although the addition of pairs of nitrogenous compounds, such as glutamine and aspartic acid as well as asparagine and glutamic acid, could result in considerable increases in the yields of inosinic acid, this was not the case when (1) glutamic acid and glutamine, (2) aspartic acid and asparagine, or (3) aspartic acid and glutamic acid were combined. It is thus apparent that both a four-carbon and a five-carbon dicarboxylic acid are necessary in the synthesis and that at least one of these must have an amide linkage.

In a study of compounds which could behave as metabolic inhibitors of purine synthesis *de novo*, the compound L-azaserine was tested. This new antibiotic, which may cause the regression of certain tumors when administered to animals (33), causes a drastic reduction of purine synthesis *de novo* when added to vessels containing asparagine and glutamic acid in addition to the materials included in the control vessels. The results of this experiment are illustrated in the final bar of Fig. 2.

#### *Role of glycine derivatives in the purine biosynthetic mechanism.*

In an effort to discover the nature of some of the possible intermediates prior to the formation of 5-amino-4-imidazolecarboxamide ribotide in the synthesis *de novo* of inosinic acid from glycine, Brooks, Schulman, Williams, Levenberg, and Buchanan (9) have synthesized a variety of analogues of the latter amino acid labeled with radioactive carbon and have tested them *in vitro* in a pigeon liver homogenate system or *in vivo* by intraperitoneal injection in rats. The structures of these compounds are given in Fig. 3.

In no instance could evidence be obtained to indicate that compounds (II), (III), (IV), or (V) were efficient precursors of nucleic acid adenine or guanine or urinary allantoin in the intact rat. In the purine biosynthetic system of pigeon liver, compounds (I), (II), (III), or (IV) were far less efficient precursors of inosinic acid than glycine-1-C<sup>14</sup>, and in all cases where isotope was incorporated into the purine structure, a bank of unlabeled glycine present in the vessel together with the isotopic test compound resulted in marked



reduction in the incorporation of the label into inosinic acid. It thus is evident that the small amount of inosinate synthesis resulting from the isotopic test compounds occurred by virtue of the fact that the latter materials were degraded enzymatically to glycine and thence diluted by the bank of this amino acid prior to utilization in purine biosynthesis *de novo*.

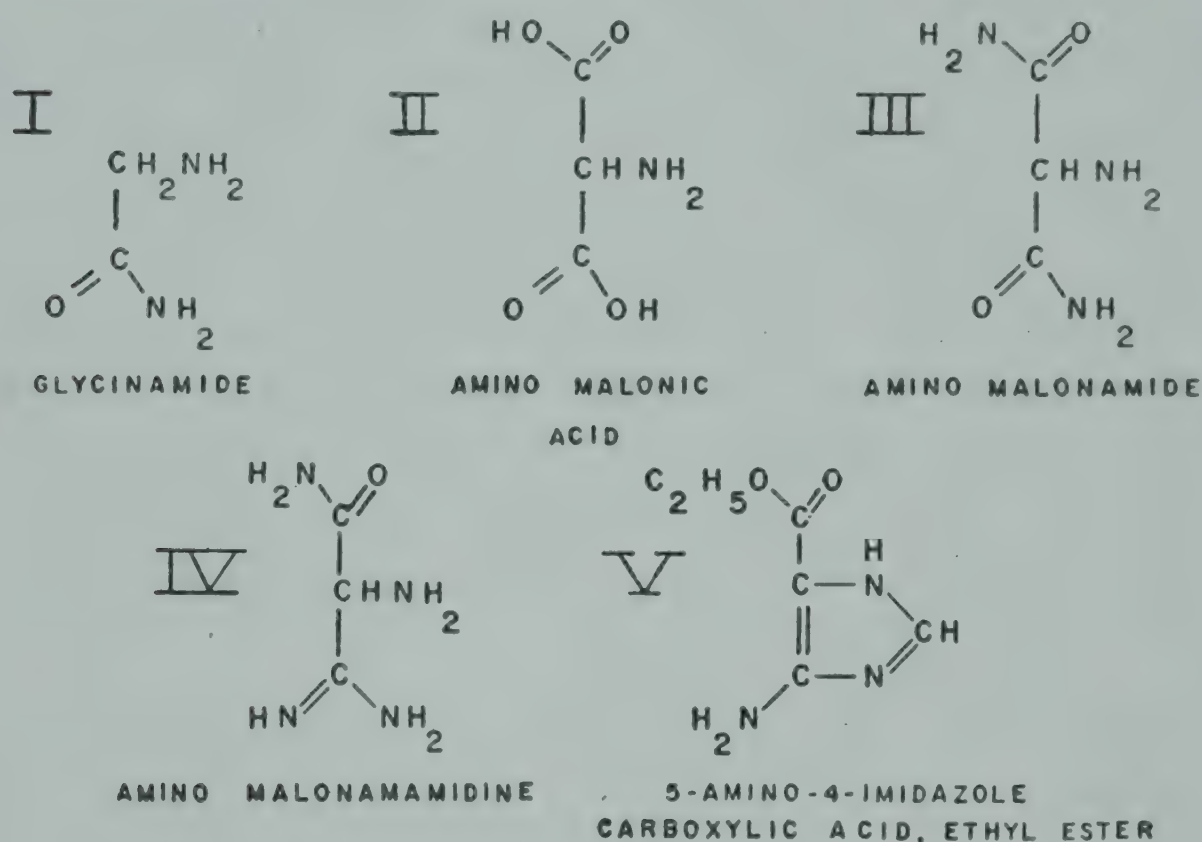


FIG. 3. Structures of the compounds tested as purine precursors.

The above experiments imply that glycinamide, amino malonic acid, amino malonamide, and amino malonamidine, *per se*, are not intermediates in the long sequence of enzymatic reactions converting glycine, formate, bicarbonate, and dicarboxylic amino acids and amides to inosinic acid. It is conceivable, however, that these compounds, just as in the case of 5-amino-4-imidazolecarboxamide, are true intermediates in this biosynthetic pathway only when in the form of their corresponding acyclic (N-amido) ribotides. Of particular interest in this respect is the recent report (13) of the tentative identification of an acyclic ribotide of glycinamide accumulating in purine-synthesizing systems supplemented with glycine, glutamine, and ribose-5-phosphate but deprived of bicarbonate and formate.

This finding implies that ribotidation must occur at a very early stage in the metabolic conversion of glycine to an intact purine structure, but from the above observations the sequence glycine  $\rightarrow$  glycinamide  $\rightarrow$  glycinamide ribotide appears unlikely.

### THE ROLE OF AMINO ACIDS IN TRANSFORMYLATION REACTIONS OF PURINES

As mentioned in the previous section, the synthesis de novo of a purine ring requires as carbon atom precursors the compounds glycine, formate, and  $\text{CO}_2$  in the ratio of 1:2:1. An additional fact of importance was discovered in 1951 by G. Robert Greenberg (14), who at Western Reserve University provided evidence that inosinic acid was formed from radioactive purine precursors. He incubated radioactive formate with pigeon liver homogenates and isolated radioactive products of the reaction by means of paper chromatographic methods. One product of the reaction, inosinic acid, had a higher specific activity than either inosine or hypoxanthine, a fact indicating that it is a metabolic precursor of the latter compounds. These results imply that at some stage in the long sequence of reactions leading to an intact purine—that is, a stage where the ring has not yet been fully formed—a ribosyl-5-phosphate moiety was added to a purine precursor. The nature of this step is not known, but present indications are that it occurs very early in the sequence of reactions, possibly at a level involving an amino acid or its derivative.

#### *The exchange reaction of formate with the 2-position of inosinic acid.*

In an attempt to confirm the above findings by a different experimental procedure, Schulman and Buchanan (28) incubated non-isotopic inosinic acid, inosine, and hypoxanthine with pigeon liver homogenates which contained the substrates of synthesis de novo. One of the substrates, either glycine or formate, was radioactive.

The specific activities of the isolated purine compounds provided confirmation of the findings of Greenberg that inosinic acid was the precursor of either inosine or hypoxanthine. However, the additional



observation was made that the ratio of incorporation of formate: glycine was in many cases greater than the expected 2:1 (7). Degradation of uric acid obtained from this inosinate by a procedure capable of separating carbon atoms 2 and 8 of the purine ring showed that most, if not all, of the extra radioactivity was in the 2 position of the ring. It thus appeared that inosinic acid could undergo reactions by which the carbon atom of position 2 could be exchanged with radioactive formate. This reaction was designated as the "formate exchange" reaction, and the enzyme system responsible for the reaction was designated as *inosinic acid transformylase*.

Apparently this exchange reaction occurred only in the presence of inosinic acid. Hypoxanthine and inosine were not capable of substituting for the nucleotide. Since the amount of formate incorporated by the exchange reaction is small when compared to that which could be utilized in synthesis *de novo*, an attempt was then made to limit synthesis *de novo* by the omission of one specific precursor, in order that the exchange reaction be made more apparent. Since  $\text{CO}_2$  is the specific precursor of position 6, bicarbonate was omitted from the incubation medium. Under these conditions the exchange reaction is very evident.

As the citrovorum factor (CF) has been implicated in the metabolism of "one-carbon" compounds (19, 36), its effects in this system were studied, and a stimulation of formate exchange was noted. This was the first demonstration of the effect of the citrovorum factor on a specific enzymatic reaction in a cell-free system. The results of some experiments demonstrating these points are shown in Table 3. A point worth noting here is that omission of bicarbonate from the incubation vessels brings out the marked stimulatory effect of CF on the exchange reaction.

To delineate the factors involved in the reaction, the enzymes of the soluble extract were precipitated by addition of ethanol to a concentration of 45 per cent at low temperatures. A comparison of the exchange reaction with the soluble extract and with the ethanol precipitated fraction is shown in Table 4 (11). Two types of results were obtained. By far the more frequently observed were those of

TABLE 3

EFFECT OF LEUCOVORIN AND CO<sub>2</sub> ON THE FORMATE EXCHANGE REACTION  
IN EXTRACTS OF PIGEON LIVER †

Vessel *	Leucovorin Present or Absent	CO <sub>2</sub> Present or Absent	Radioactive Substrate Incorporated μM./mM. Inosinic Acid		
			Glycine Position 4	Formate Position 2	Position 8
1	—	+	18.8	21.2	14.9
2	+	+	23.8	36.5	16.9
3	—	—	0.54	3.48	0.98
4	+	—	1.14	9.10	1.86

\* Each vessel represents a pair of vessels, one of which contains HCOONa-C<sup>14</sup> and glycine giving incorporation into positions 2 and 8, the second contains HCOONa and glycine-1-C<sup>14</sup> giving incorporation into position 4.

The vessels each contained, in a total volume of 2.6 ml., glycine 20 μM., HCOONa 20 μM., Na-inosinate 8 μM., and 2.0 ml. pigeon liver extract (equivalent to 0.8 g. liver). Where added, bicarbonate was 90 μM. and leucovorin 60 γ.

The vessels were incubated for 6 minutes at 38° C. and the reaction stopped by boiling for 3 minutes. The inosinic acid was isolated on a Dowex-1-Cl column and converted to uric acid, which was counted directly in the case of the glycine-1-C<sup>14</sup> vessels, and degraded to separate positions 2 and 8 in the case of the formate vessels.

† From Buchanan and Schulman (7).

TABLE 4

FORMATE EXCHANGE REACTION WITH PRECIPITATED ENZYMES

Vessel *	System †	Experiment 1 Radioactive Substrates Incorporated μM./mM. Inosinic Acid		Experiment 2 Radioactive Substrates Incorporated μM./mM. Inosinic Acid
		Glycine	Formate	Formate
1	Soluble Extract	0.78	3.13	—
2	0-45% EtOH Fraction	0	0	1.46
3	0-45% EtOH Fraction + boiled juice	0.30	4.33	1.07
4	0-45% EtOH Fraction + boiled juice + leucovorin	2.10	10.24	3.31
5	0-45% EtOH Fraction + leucovorin	0	0	0.23

\* Each vessel in the table represents a pair of vessels, as in Table 1.

† Bicarbonate is omitted in these vessels.

The vessels each contained, in a total volume of 1.5 ml., glycine 9.0 μM., HCOONa 9.0 μM., Na-inosinate 4.5 μM.; leucovorin 38 γ, as indicated.

The incubation, isolation, and counting procedures are the same as Table 1.



Experiment 1. Here there was no incorporation of radioactive formate into inosinic acid unless an extract of pigeon liver boiled juice is added to the system. Upon addition of leucovorin and pigeon liver boiled juice extract, a further increase in incorporation is shown, whereas addition of leucovorin by itself resulted in no incorporation of radioactive substrates. It would seem that the results obtained with the reconstituted system approximate closely those obtained with the soluble extract, as shown in Table 3.

However, in occasional experiments it was noted, as shown in Experiment 2 of Table 4, that incorporation of formate would occur with the 0-45 per cent ethanol fraction in the absence of any additional cofactors. The addition of boiled juice in these experiments brought about a depression in incorporation, but a stimulation could be shown upon the addition of leucovorin in the presence of boiled juice.

In order to explain these conflicting results and to eliminate synthesis *de novo*, recourse was made to further fractionation. Two fractions, precipitable with ethanol, have been obtained, which while carrying out the same overall reaction, behave differently with respect to additional factors. The results of an experiment with these two fractions is shown in Table 5.

Fraction I, which precipitates between 15 and 30 per cent ethanol, shows a marked incorporation of radioactive formate in the absence of any additional factors. Addition of boiled juice to this fraction results in an almost complete elimination of formate incorporation. Upon addition of leucovorin, in the presence of boiled juice, there is some increase in incorporation over that observed with boiled juice alone, but the activity is never as great as the enzyme fraction without any additions.

Fraction II, which precipitates between 25 to 45 per cent ethanol, shows no incorporation of radioactive formate unless boiled juice is added. Upon the addition of leucovorin, in the presence of boiled juice, there is a reduction in the formate incorporated.

With either fraction in this experiment there is almost no incorporation of radioactive glycine, a result which indicates that synthesis



TABLE 5  
FORMATE EXCHANGE REACTION IN FRACTIONATED SYSTEM

Vessel *	System	Radioactive Substrates Incorporated $\mu\text{M.}/\text{mM.}$ Inosinic Acid	
		Formate	Glycine
1	Fraction I	7.08	0.00
2	Fraction I + boiled juice	0.26	0.01
3	Fraction I + boiled juice + leucovorin	1.26	0.08
4	Fraction II	0.01	0.00
5	Fraction II + boiled juice	4.33	0.04
6	Fraction II + boiled juice + leucovorin	2.84	0.00

\* Each vessel in the table represents a pair of vessels, as in Table 1.

Fraction I = 15-30% ethanol-precipitated fraction

Fraction II = 25-45% ethanol-precipitated fraction

The experimental conditions are exactly the same as in Table 2.

de novo has been eliminated. However, it has been found in subsequent experiments that while glycine is not incorporated as such, the addition of glycine to the reaction mixture in proper concentration gives a definite stimulation to formate incorporation. No other amino acid tested thus far has been able to show this effect, but in contrast, serine has been shown to inhibit formate incorporation markedly.

The data presented thus far on the exchange reaction requirements suggest that the reactions which lead to the incorporation of formate into position two of inosinic acid involve the following steps: (1) cleavage of the purine ring; (2) equilibration of these reaction products with radioactive formate; and (3) re-formation of the purine ring with inclusion of radioactive formate in the 2 position.

In addition, the previously cited effects of glycine and serine implicate their metabolism in this reaction sequence. Since the net result measured, i. e., the incorporation of formate into inosinic acid, is the result of a balance between a complex series of reactions, it was decided that this approach would not lend itself easily to further elucidation of the reaction sequence.



*The Formation of 5-Amino-4-imidazolecarboxamide Ribotide from Inosinic Acid.*

Cleavage of the purine ring of inosinic acid and transfer of the formyl group implies the formation of 5-amino-4-imidazolecarboxamide ribotide.\* This compound possesses an aryl amine, and like the free base and riboside, shows a positive Bratton-Marshall diazo reaction. Fraction I, the 15-30 per cent ethanol fraction, which does not require any additional cofactors, was incubated with Na-inosinate and a large amount of glycine. The incubation products were chromatographed on a Dowex-1-Cl column using 0.003 *N* HCl for elution, and a fraction was obtained which showed a ratio of aryl amine : pentose : organic phosphate of approximately 1:1:1. The compound also possessed an ultraviolet absorption peak at 267 *mμ* similar to that of the free base (12).

TABLE 6

FORMATION OF 5-AMINO-4-IMIDAZOLECARBOXAMIDE RIBOTIDE BY FRACTION I\*

System	$\mu$ M. Aryl Amine
Fraction I + inosinate	0.000
Fraction I + glycine	0.000
Fraction I + leucovorin	0.010
Fraction I + boiled juice	0.00
Fraction I + inosinate + glycine	0.065
Fraction I + inosinate + glycine + leucovorin	0.166
Fraction I + inosinate + glycine + boiled juice	0.057
Fraction I + inosinate + glycine + boiled juice + leucovorin	0.328

The substrates, where added, in a total volume of 1.0 ml., were: Na inosinate 10  $\mu$ M., glycine 35  $\mu$ M., leucovorin 50  $\gamma$ , boiled juice 0.2 ml. from 1:1.5 pigeon liver homogenate.

0.5 ml. enzyme were added to each vessel representing 30 mg. of lyophilized Fraction I taken up in 0.1 *M.* Tris buffer, pH 7.4.

Vessels incubated for 25 minutes at 30° C. and reaction stopped by the addition of 0.5 ml. 30% TCA.

\* From Flaks and Buchanan (12).

\* The addition of a ribose phosphate moiety to the imidazolecarboxamide group necessitates numbering the compound as 5-amino-4-imidazolecarboxamide ribotide. The free base is commonly designated 4-amino-5-imidazolecarboxamide.

If glycine is acting in this system as a formyl acceptor, one should obtain increased formation of 5-amino-4-imidazolecarboxamide ribotide by increasing the concentration of glycine. It has been possible to demonstrate this. A similar increase in formation of 5-amino-4-imidazolecarboxamide ribotide has been obtained upon elevating the concentration of inosinic acid. As with the exchange reaction, no other amino acid except glycine can stimulate the reaction.

The effect of additional factors upon the reaction catalyzed by Fraction I has been studied, and the results are shown in Table 6. In the exchange studies the addition of boiled juice to this fraction caused a marked depression in formate incorporation. With the carboxamide ribotide formation there is no effect. The addition of leucovorin or anhydroleucovorin causes a stimulation of carboxamide ribotide formation, and the addition of boiled juice to the system in the presence of leucovorin gives a further doubling in carboxamide ribotide formation. It was subsequently found that the boiled juice could be replaced by an equivalent of an ashed preparation of boiled juice, and this in turn by  $\text{Cu}^{++}$  at a level of  $10^{-4}$  M. Further confirmation of a role of  $\text{Cu}^{++}$  here has come from the finding that the stimulation obtained by the presence of  $\text{Cu}^{++}$  and leucovorin is eliminated by adding to the incubation system either  $\text{CN}^-$  or versene, both of which are powerful  $\text{Cu}^{++}$ -complexing agents.

Studies on the reverse reaction, the conversion of 5-amino-4-imidazolecarboxamide ribotide to inosinic acid, have also been carried out. Fig. 4 shows the results of an experiment in which the carboxamide ribotide was allowed to accumulate during the first 10 minutes of the reaction by the incubation of Fraction I with 5  $\mu\text{M}$ . of glycine and 10  $\mu\text{M}$ . of inosinate. At the 10-minute point various additions were made to separate vessels, and the reaction was run for an additional 15 minutes. With no further additions there is a further small increase in carboxamide ribotide. Upon addition of 50  $\mu\text{M}$ . of glycine, the formation of carboxamide ribotide continued almost linearly up to the conclusion of the experiment. The addition of either 50  $\mu\text{M}$ . of sodium formate or 50  $\mu\text{M}$ . of N-formylglycine caused little disappearance of carboxamide ribotide. However, the



addition of 50  $\mu$ M. of L-serine caused a rapid and complete disappearance of the ribotide.

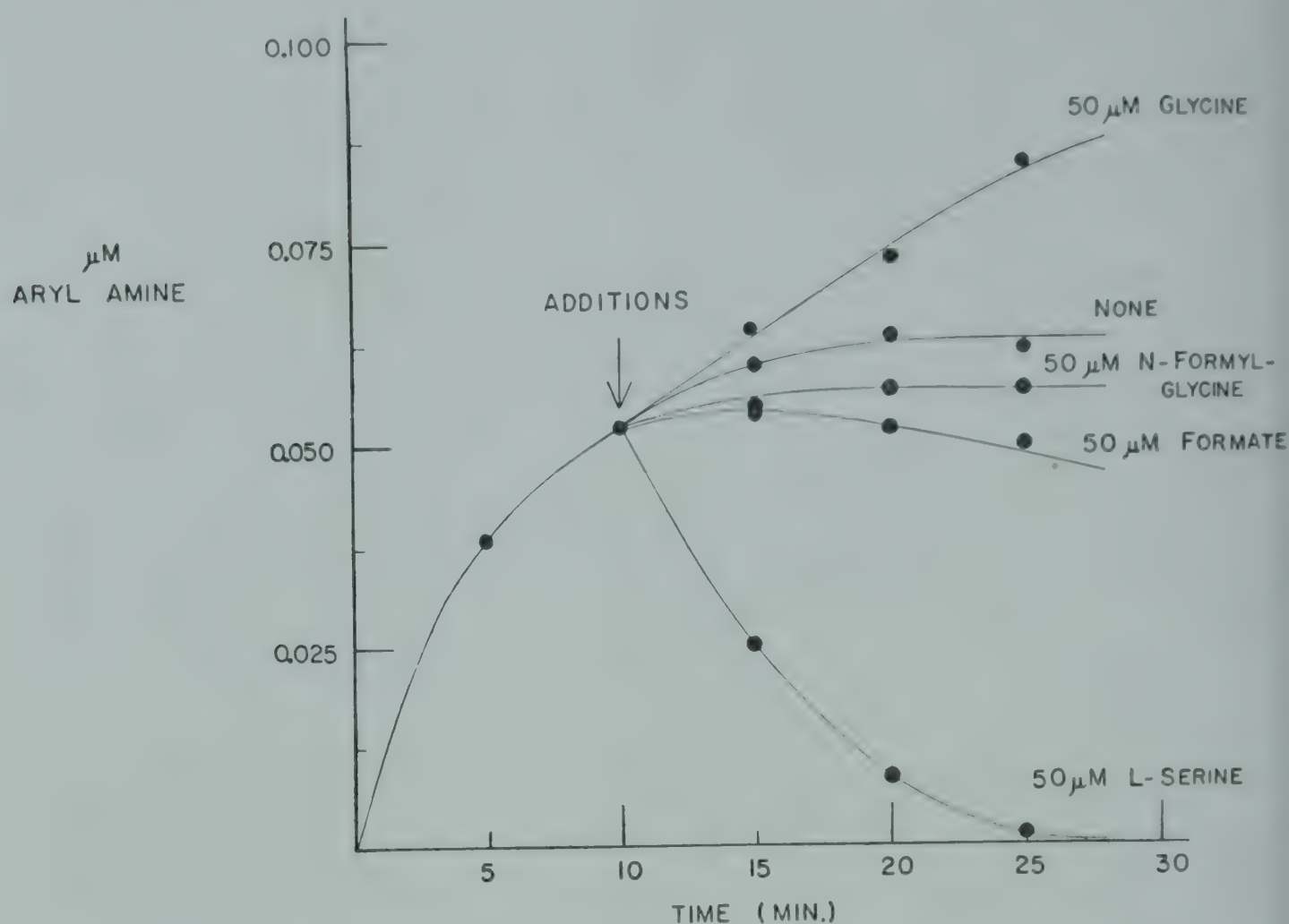


FIG. 4. Effect of various compounds on 5-amino-4-imidazolecarboxamide ribotide formation and disappearance. Additions were made to separate vessels at the 10-minute point except the vessel marked "None."

### SUMMARY

A summary of the events occurring in this system is given in Fig. 5. The ring of inosinic acid is first presumed to open between positions 1 and 2 so as to yield 5-formamido-4-imidazolecarboxamide ribotide, a compound which has not yet been isolated. In the presence of the transformylase the formyl group is transferred to enzyme-bound transformylating cofactor (CoF) yielding enzyme-bound formyl CoF and 5-amino-4-imidazolecarboxamide ribotide. The exchange reaction would then occur between radioactive formate and enzyme-bound formyl-CoF. Then, by reversal of the sequence of reactions up to this point, inosinic acid-2- $C^{14}$  would be formed.

In the presence of a suitable reducing agent the enzyme-formyl-

CoF could form serine in the presence of glycine, since the conversion of position 2 of inosinic acid to the  $\beta$ -carbon of serine would require a one-step reduction. In all the experiments carried out in this laboratory the formation of carboxamide ribotide is relatively small, and the limiting factor here could very well be the appropriate reductive source. As yet there is no available information on this point.

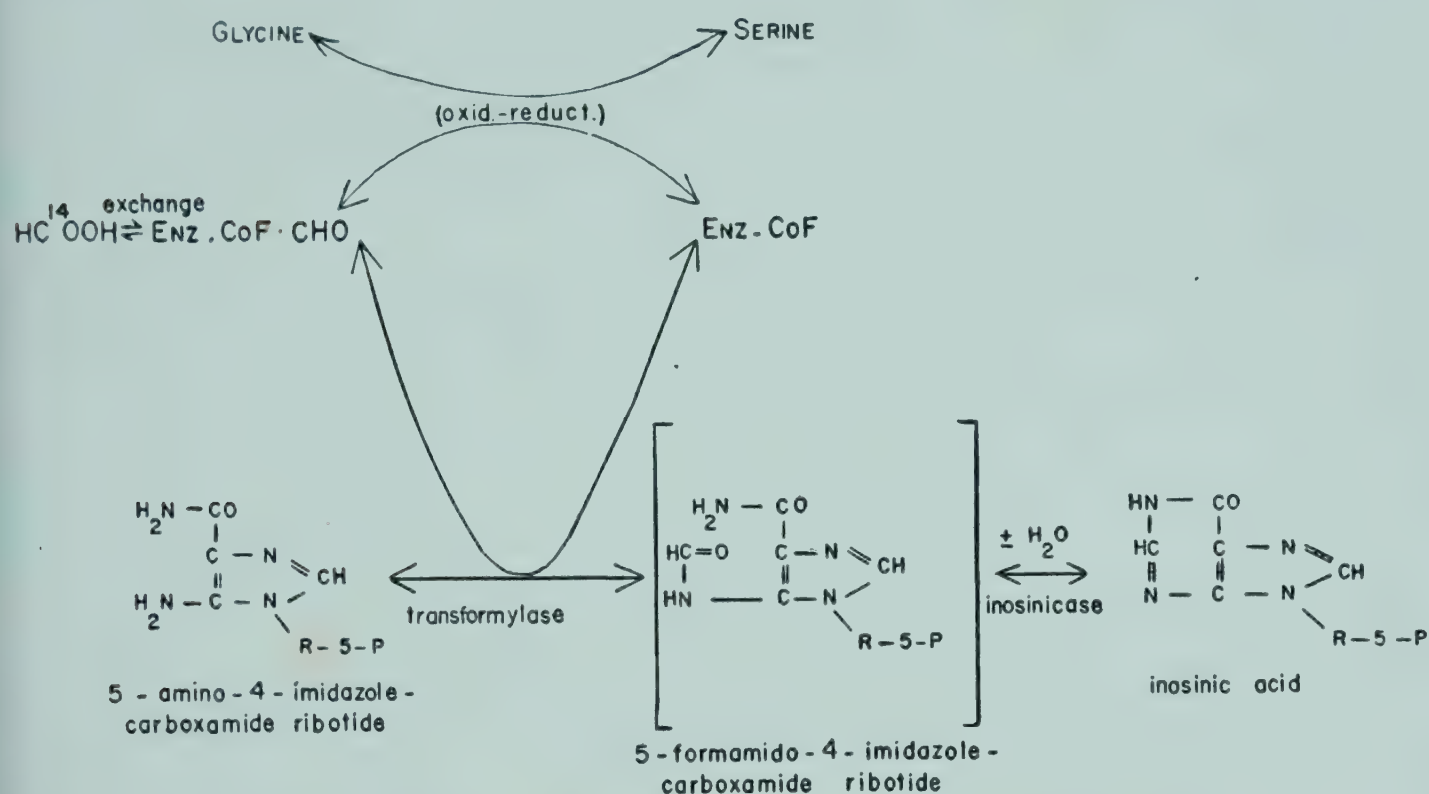


FIG. 5. Reactions involved in the formation and disappearance of 5-amino-4-imidazolecarboxamide ribotide, the exchange reaction, and the interrelating glycine-serine conversion.

An important problem is the major metabolic source of one-carbon compounds in animal metabolism. Sidney Rieder (26) has carried out experiments with glucose-1- $\text{C}^{14}$  which give information on this point. He has found that uric acid, highly labeled in positions 2 and 8, is excreted by chickens fed glucose-1- $\text{C}^{14}$ . Since labeling of carbon 1 of glucose could theoretically give rise to  $\beta$ -labeled serine, it is possible that the  $\beta$ -carbon of serine, and indirectly the carbon atoms of glucose, are the primary source of the reduced one-carbon compounds utilized in purine biosynthesis.

A further point should be mentioned regarding the scheme. No role has been given to the  $\text{Cu}^{++}$  and leucovorin effects noted nor



has the CoF been identified. This is due to the fact that a second enzyme fraction, Fraction II, mentioned in the exchange studies, is also capable of carrying out the conversion of inosinic acid to carboxamide ribotide. This enzyme fraction has an absolute dependency for a heat-stable factor present in boiled juice, which is exactly the same case when the exchange reaction is carried out with this fraction. The formation of carboxamide ribotide by this enzyme fraction and its cofactor shows the same specific requirements for glycine as when the reaction is catalyzed by Fraction I. Whereas the reaction as catalyzed by Fraction I is stimulated by the addition of anhydroleucovorin or leucovorin and  $\text{Cu}^{++}$ , this does not occur with Fraction II.

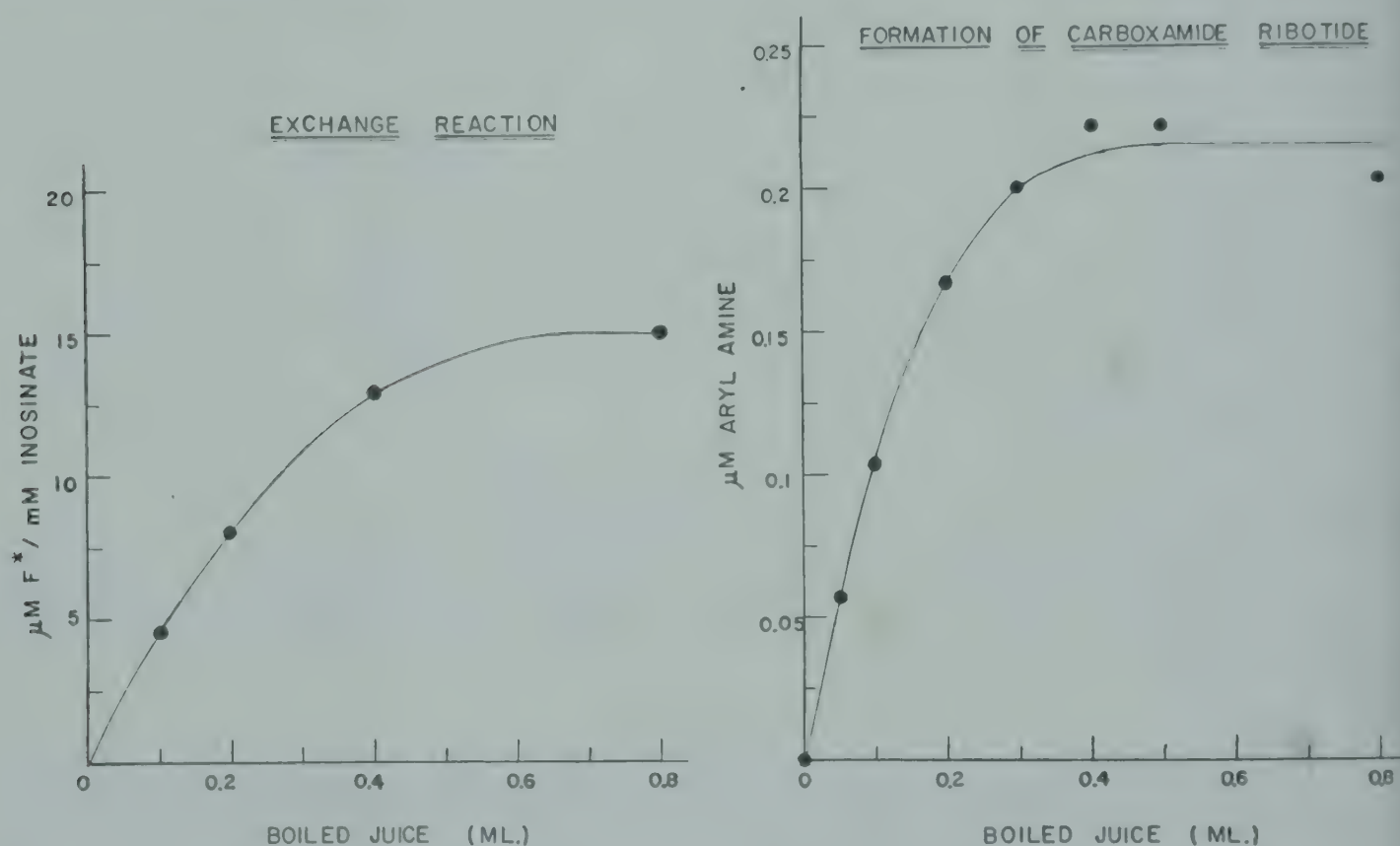


FIG. 6. Activity of boiled juice factor with Fraction II in the exchange reaction and 5-amino-4-imidazolecarboxamide ribotide formation.

It is our present belief that Fraction II contains the apoenzyme related to the holoenzyme present in Fraction I. Further it is believed that the active formylating cofactor, CoF, is identical with a factor present in boiled juice which stimulates the reaction as catalyzed by Fraction II. This view is supported by the observation that boiling of Fraction I, followed by removal of denatured protein, yields a clear extract capable of activating Fraction II.

The activity of Fraction II merits further discussion. Since there is no activity in either the exchange reaction or the formation of carboxamide ribotide in the absence of the factor, it has been possible to show a linear increase in activity with increases in the amount of the boiled juice factor until saturation of the system occurs. The results of such experiments are shown in Fig. 6. It can be seen from the two graphs in the figure that optimal activity for both the formate exchange reaction and formation of carboxamide ribotide occurs at similar levels of the added boiled juice factor.

Reports have appeared in the literature regarding the nature of the formylating cofactor in a variety of systems. Thus Greenberg (15, 16) has stated that enzymes from pigeon liver can carry out the conversion of tetrahydrofolic acid or leucovorin, in the presence of ATP, to a compound possessing properties of a transformylating cofactor. Similarly, Kisliuk and Sakami (21) and Blakely (3) have stated that tetrahydrofolic acid is or may be converted to a transformylating cofactor active in serine biosynthesis.

In one of our systems, as mentioned above, leucovorin or anhydro-leucovorin in the presence of  $\text{Cu}^{++}$  has the ability to stimulate the reaction, but in another system which carries out the same reaction neither of the above is capable of replacing a factor present in a boiled extract of beef or pigeon liver. The identification of the exact chemical composition of the transformylating cofactor will depend in part on the development of a test system composed of relatively purified enzymes. At present one cannot decide from the many effects of pteroyl compounds in vitro, which one is the active agent per se, and which compounds are converted to the active agent by enzymes of impure systems used to test their action.

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# THE BIOSYNTHESIS OF THE PURINE RING

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AMINO ACIDS play an important role in the biosynthesis of many of the more complex molecules. The synthesis of the purine ring provides an excellent example of this, since the four nitrogens and probably four out of the five carbons arise from amino acids. Because a variety of reaction mechanisms are involved in the biosynthesis of purines, it is conceivable that some of these may participate in other pathways.

The problem of the biosynthesis of purine ring can be divided reasonably into two areas. The first is concerned with the source of the carbons and nitrogens which make up the ring, and the second with the mechanisms by which these are assembled to form the ring. The experimental work which led to the identification of formate as a source for carbons 2 and 8 (36),  $\text{CO}_2$  for carbon 6 (4), glycine for carbons 4 and 5 (4) and nitrogen 7 (31), glutamine for the nitrogens 3 and 9, and glutamic or aspartic acid for the nitrogen 1 (37) was essential before a direct approach to the mechanisms of the synthetic reactions could be attempted. However, it should be pointed out that some of these compounds may not represent the direct precursors, but only the class of compounds involved. For example, while formate enters the 2 and 8 positions of the purine ring very readily in vivo, serine (9) and  $\delta$ -aminolevulinic acid (32) have been suggested as natural precursors. Likewise, the direct precursor of carbon 6 is unknown. Obviously, knowledge of the immediate precursors depends upon exact knowledge of the intermediates involved in these reactions, and definitive knowledge concerning these is sparse. However, there are a few pieces available for the construction of this puzzle, and these are illustrated in Fig. 1.



The initial stumbling block in the elucidation of purine biosynthesis was the concept that the purine base was synthesized *per se* and not as a derivative. The demonstration that inosinic acid (V) was the precursor of hypoxanthine in a system *in vitro* (14) opened the door for the consideration of nucleotides as early precursors. It was reasoned that 5-amino-4-imidazolecarboxamide (34), which accumulates in sulfonamide-inhibited cultures of *Escherichia coli* (38),

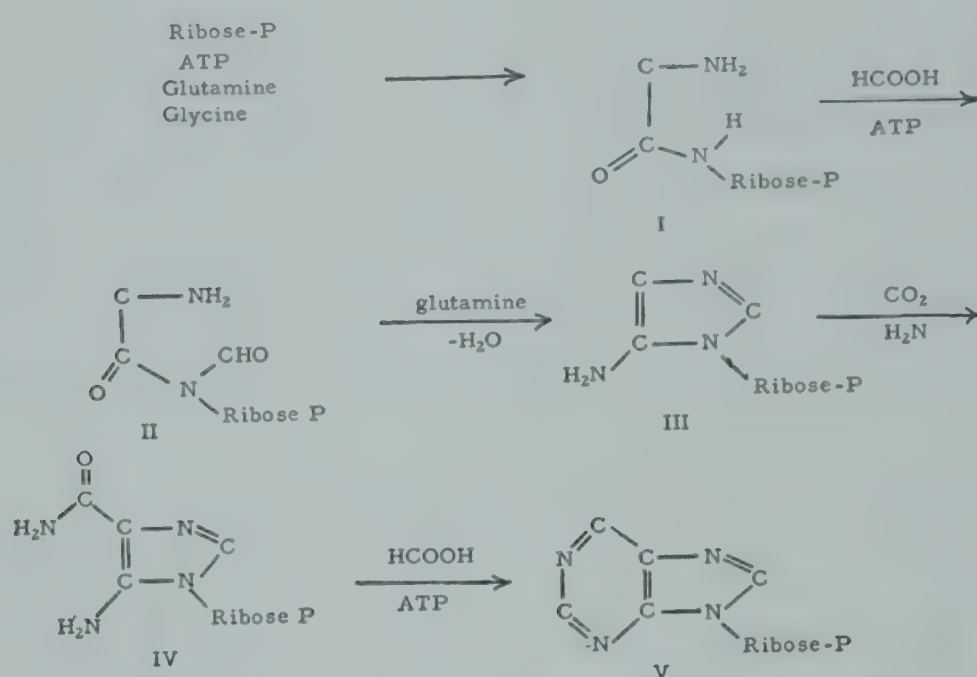


FIG. 1. Proposed Scheme for Purine Biosynthesis.  
(The position of the CHO group in II is unknown.)

should accumulate as the nucleotide derivative, and this concept was supported by the isolation of the carboxamide riboside (15) and ribotide (IV) (16). It has since been shown that the carboxamide ribotide accepts an "activated" formyl group to give inosinic acid (17). Recently the accumulation in biotin-deficient yeast of an aryl amine which will replace the adenine requirement of another strain has been reported (5), and this compound may be related to a proposed aminoimidazole ribotide (III) isolated from the culture medium of a purine-requiring *E. coli* strain (5). Finally, the isolation of two glycine ribotide compounds (I, II) makes it possible to draw the tentative biosynthetic scheme presented in Fig. 1. The glycine ribotides, the mechanism of fixation of formate into carbons 2 and 8, and the possible mechanism for the fixation of CO<sub>2</sub> will be discussed.

## GLYCINE RIBOTIDE PRECURSORS

The initial detection of the glycine ribotide compounds was the result of several observations. In an acetone powder extract of pigeon liver, supplemented as indicated in Table 1,  $C^{14}$ -formate

TABLE 1

$\mu M.$   $C^{14}$ -FORMATE INCORPORATED INTO PURINE AND NON-PURINE FRACTIONS  
BY PIGEON LIVER ACETONE POWDER EXTRACT

	Non Purine (a)	Purine 2 and 8 (b)	Ratio a/b
Complete System	.204	.147	1.4
$HCO_3^-$ omitted	.145	.015	9.7

System: 0.4 ml. of 1/10  $K_2HPO_4$  (0.05  $M.$ ) extract, IMP 10  $\mu M.$ , ATP 5  $\mu M.$ , 3-phosphoglyceric acid 15  $\mu M.$ ,  $KHCO_3$  7  $\mu M.$ ,  $MgCl_2$  8  $\mu M.$ , homocysteine 16  $\mu M.$ , pigeon liver boiled extract (1 : 2) 0.2 ml.,  $C^{14}$ -formate 5  $\mu M.$  Total vol., 0.65 ml.

activity was found not only in carbons 2 and 8 of purine, but also in a non-purine fraction. The omission of  $CO_2$  from the system resulted in a marked decrease of formate fixation into purines, but only a slight decrease in the activity of the non-purine fraction (29). The  $C^{14}$ -moiety of the latter fraction was found to be easily hydrolyzed (1  $N$  HCl 100° C., 10 min.) to a volatile form. Likewise, in a similar system total glycine fixation was noted to be 3 to 4 times the amount utilized in the synthesis of purines de novo. Comparable mixtures were incubated with either  $C^{14}$ -formate or glycine-1- $C^{14}$  and the trichloroacetic acid filtrates were subjected to two-dimensional paper chromatography. Radioautograms indicated that  $C^{14}$ -glycine was fixed into several compounds which appeared to be identical with the  $C^{14}$ -formate compounds and which moved as nucleotides. Preliminary experiments indicated that a mixture of these compounds served as a precursor of inosinic acid.

For large-scale preparations of the precursors (13), the acetone powder extract was concentrated by lyophilization. Glycine-1- $C^{14}$  was employed, since the isolation of the intermediates depended



upon their radioactivity. Recently, in experiments with a Dowex-treated and dialyzed extract, supplemented with citrovorum factor, ATP,<sup>1</sup> 3-PGA, and C<sup>14</sup>-formate, the requirements for glycine, glutamine, and ribose-5'-phosphate have been demonstrated (Table 2).

TABLE 2  
EFFECT OF SUBSTRATE ON FORMYL GLYCINE RIBOTIDE FORMATION

Omission	Hydrolyzable C <sup>14</sup> -Formate, $\mu$ M.	Non-hydrolyzable C <sup>14</sup> -Formate, $\mu$ M.
None	.488	.009
Glycine	.044	.008
Glutamine	.081	.033
Ribose-5-P	.124	.016

Citrovorum factor (CF) and ATP are also necessary constituents of the reaction system. Preliminary fractionation studies indicated that the enzyme system could be precipitated with 20 per cent ethanol.

The initial step in the isolation of these compounds involved the chromatography of the trichloroacetic acid filtrate on thick papers with a butanol-acetic-acid solvent system. The mixture of radioactive compounds which moved as nucleotides was resolved on a Dowex-1 formate column, as indicated in Fig. 2.

Compound I on elution at pH 5.2 could be separated with difficulty into two components. Glycine-1-C<sup>14</sup>, but not C<sup>14</sup>-formate, was incorporated into these components.<sup>2</sup> Compound II could be separated with relative ease at pH 5.0 into 2 fractions which can be labeled with either C<sup>14</sup>-glycine or C<sup>14</sup>-formate. Acid will catalyze the interconversion of these fractions. For example, if the fraction giving rise to the first peak is allowed to stand at 0° C., pH 1.0, for 48 hours

<sup>1</sup> Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; IMP, inosinic acid; IRMP, 5-amino-4-imidazole-carboxamide-5'-phosphoriboside; CF, citrovorum factor (N<sup>5</sup>-formyl-5, 6, 7, 8-tetrahydrofolic acid); FA, folic acid; FAH<sub>2</sub>, dihydrofolic acid; FAH<sub>4</sub>, tetrahydrofolic acid; FAH<sub>4</sub>CHO, formyltetrahydrofolic acid, exact structure unknown; ACF, anhydroleucovorin; DPN, diphosphopyridine nucleotide; 3-PGA, 3-phosphoglyceric acid.

<sup>2</sup> These data are obtained with a 32 cm.  $\times$  1.76 cm.<sup>-2</sup> column. With a small column, I may be eluted at pH 6.5 as a single peak.

and is then rechromatographed, one obtains two peaks. The same can be shown with the other fraction. This interconversion as well as the analytical data (see Table 4) suggest that the two forms of II are isomers (a and b). The nature of the isomerization is not known.

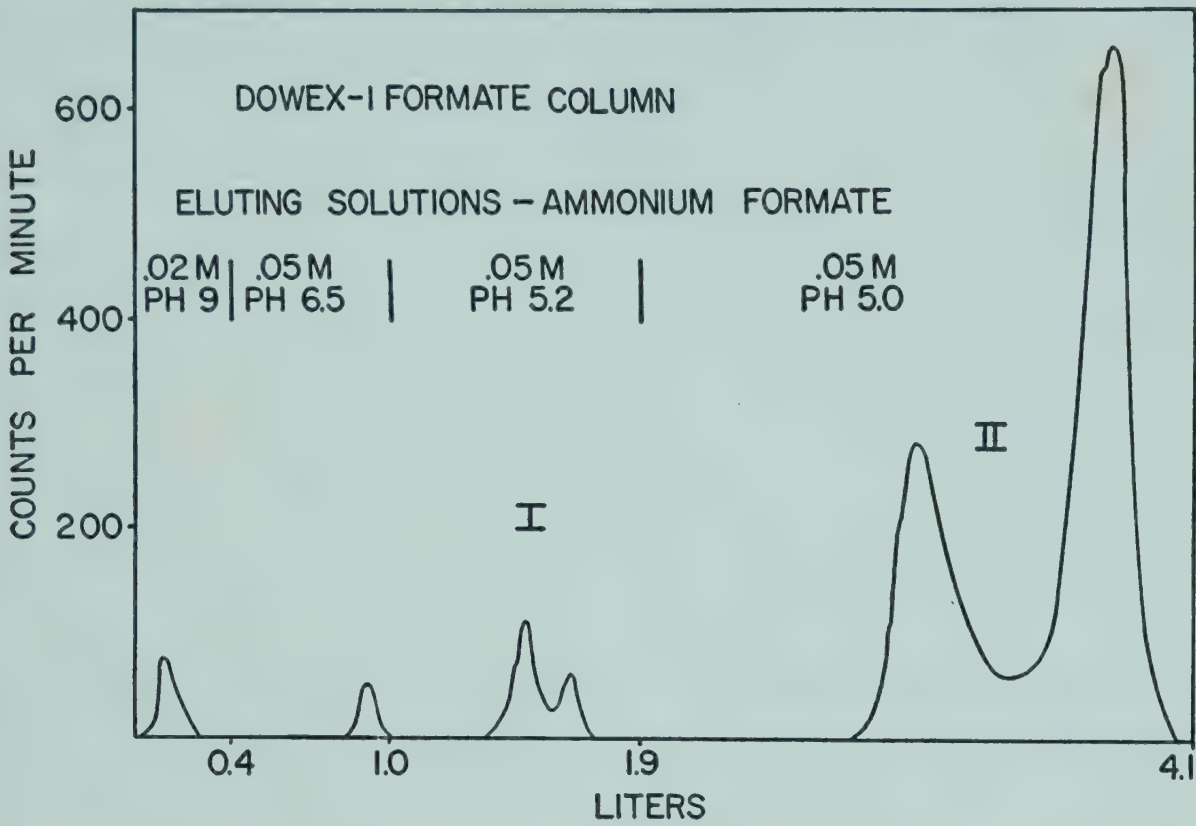


FIG. 2.

These compounds serve as precursors of purine in the pigeon liver system, which synthesizes inosinic acid and hypoxanthine (Table 3).

TABLE 3  
GLYCINE RIBOTIDES AS PURINE PRECURSORS

Precursor	C <sup>14</sup> Label	μM. in Hypoxanthine
Compound I	glycine	.014
Compound II	glycine	.035
Glycine	—	.013
Compound II	Formate	.015
Formate	—	.070

Compound II, when labeled with either glycine-1-C<sup>14</sup> or C<sup>14</sup>-formate, transfers this label to the purines, and the degree of incorporation is comparable to that seen in the synthesis de novo from glycine. The high level of C<sup>14</sup>-formate incorporation may represent some



exchange in the 2 position. Since the compounds were incubated in the presence of pools of unlabeled glycine and formate at levels 40 times the amount of the precursor, it is concluded that they did not break down to  $C^{14}$ -glycine or  $C^{14}$ -formate before their incorporation into purine. Degradation studies to determine the ratio of C-2/C-8/C-4 have not yet been done.

TABLE 4  
ANALYSIS OF PRECURSORS

	I	IIa	IIb
Pentose	1	1	1
Organic P	1.16	2.7	2.9
Glycine	1.28	1.45	1.40
Total N	3.7	1.75	2.30
Acid-hydrolyzable N	1.52	1.17	1.10

Preliminary analyses of these compounds are presented (Table 4). The pentose, represented as unity, is measured as orcinol-reacting material which has a spectrum identical with that obtained with ribose-5'-PO<sub>4</sub>. Desoxyribose and ketose tests were negative. Organic phosphate in compound I approaches unity. Organic phosphate in compound II has always given values above unity. The lowest value of 1.8 was obtained on material isolated by the following chromatographic steps: paper, butanol-acetic; ion exchange column, Dowex-1-formate; paper, butanol-acetic; paper, propanol-water; paper electrophoresis at pH 6.0; repeat at pH 9.3; and repeat at pH 6.0. However, for reasons to be discussed, it is believed that this compound contains only one phosphate and the excess represents impurity. Glycine values (1) are all above unity. The total N (20) above 2 probably represents an impurity, but the value of approximately 2.0 in the isomers of compound II suggests that one nitrogen exists as the amino group of glycine while the other acid-hydrolyzable nitrogen (7, 30) exists in amide linkage between glycine and ribose. The compounds do not react with ninhydrin to liberate CO<sub>2</sub> from the C-1 position of glycine or to give a color.

All of the phosphate present in compound II appears to be rela-

tively acid-stable. Hydrolysis of a preparation of compound II which contained 3  $\mu$ M. phosphate/ $\mu$ M. pentose, with 1 N HCl at 100° C. for 60 min., yielded 0.2  $\mu$ M. P/ $\mu$ M. pentose. A pyrophosphate linkage, such as that in DPN, has been ruled out by the use of prostatic phosphatase in association with HCl hydrolysis. 5'-Nucleotidase of bull semen (19) hydrolyzed the phosphate of compound II at approximately 1/50 the rate of AMP-5. On Dowex-50 hydrolysis and Dowex-1 chromatography with borate (21) compound II yielded orcinol-reacting material which migrated identically with ribose-5-phosphate, while no ribose-2- and 3-phosphates could be detected. The principal reason that compound II is believed to be a mononucleotide with one phosphate is based on considerations of its net charge. Analysis indicated two nitrogens, one of which was acid-hydrolyzable. By paper electrophoretic analysis with compounds of known charge as standards, (25) compound II showed a net charge of 0 at pH 3.6, — 0.8 at pH 7.7, — 1.2 at pH 9.0, and — 2.0 at pH 10.5. This, plus its behavior on Dowex-1 columns, favors a monophosphate structure. These charge characteristics would require that the amino group be unsubstituted, and this in turn would suggest that the formyl group is on the amide nitrogen.<sup>3</sup> The acid lability of the formyl group further suggests that the compound exists as an aliphatic ribotide. The end absorption below 240 m $\mu$  makes a conjugated double-bond system unlikely. Finally, several experiments, to be mentioned, indicate that these compounds do not contain the carbon from CO<sub>2</sub> which eventually becomes carbon 6. If compound I is a simple glycine amide ribotide, the steps leading to its synthesis may not be numerous. One would envisage the condensation of glutamine with either glycine or a ribose phosphate derivative (23, 39). A single experiment with glycine amide gave no indication that it was involved. A glutamine ribotide would seem the most logical intermediate, but there is no evidence yet for this compound.

<sup>3</sup> The position of the formyl group based on the electrophoretic mobility is in doubt since these aliphatic ribotides may not show the same mobility to charge relationships exhibited by the purine nucleotide standards.



## MECHANISM OF FORMATE ACTIVATION

A method for the study of the mechanism of activation and transfer of formate became available with the development of an acceptor system for formate, which in this case was the carboxamide ribotide. This work has been reviewed in detail elsewhere (18) and will be outlined only briefly here. In the overall reaction, 5-amino-4-imidazole carboxamide ribotide (IRMP) is converted to inosinic acid (IMP) by the introduction of a C-1 fragment which can arise from formate. The initial observation made with a pigeon liver system demonstrated the requirement for a boiled extract factor and for ATP. The overall reaction is as follows:



For each mole of diazotizable amine (IRMP) which disappeared, one mole of  $\text{C}^{14}$ -formate was fixed into a non-acid-volatile form (IMP). The IMP, which contained  $\text{C}^{14}$  activity, was isolated by column chromatography at the end of the reaction. With this formate acceptor system established, it was possible to investigate the nature of the boiled extract factor.

Since citrovorum factor (CF) had been shown to be a cofactor in the exchange reaction of formate with carbon-2 of inosinic acid (3), it seemed to be the most probable cofactor for the synthetic reaction. Indeed, it was found that boiled extract could be replaced by  $\text{CF}^4$  in Reaction 1. However, when the enzyme was diluted four-fold, boiled extract was still active while CF was inactive. The postulation that CF was converted to an active cofactor by ATP in the reaction mixture was confirmed by preincubation studies (Table 5). When CF plus ATP were preincubated for 10 min., and then IRMP and  $\text{C}^{14}$ -formate were added, and the reaction allowed to run for 5 additional minutes, 0.262  $\mu\text{M}$ . of diazotizable amine disappeared and 0.29  $\mu\text{M}$ . of  $\text{C}^{14}$ -formate was fixed. The requirement for both CF and ATP in the preincubation as well as in the overall reaction

<sup>4</sup> CF and leucovorin are used interchangeably in the text. However, in these studies the latter (a DL-mixture), was used. ACF and  $\text{FAH}_4$  were also DL-mixtures.

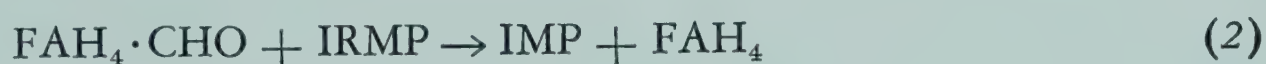
TABLE 5  
ACTIVATION OF CITROVORUM FACTOR BY ATP

	Omissions	$\Delta$ Amine $\mu M$ .	$C^{14}$ -fixed $\mu M$ .
Preincubation (10 min.)	None	.262	.294
	CF	.084	.116
	ATP	.057	.093
Total incubation (15 min.)	CF	.024	.010
	ATP	.002	0

See ref. 17.

was also demonstrated. Thus it would appear that CF is activated by ATP to form a cofactor for the introduction of formate into IRMP.

Several problems are immediately apparent. First, if this is the active cofactor, how does it function? If it functions by donating its formyl group to IRMP, then it may exist in a deformylated form ( $FAH_4$ ), and it is possible to picture the steps involved in the overall reaction (1) as follows:



The "activated" CF ( $FAH_4CHO$ ) minus its formyl group (and then denoted as  $FAH_4$ ) might be closely related to 5, 6, 7, 8-tetrahydrofolic acid. When this compound prepared by catalytic reduction of folic acid (27) was employed in the system, it was found to be active in the overall reaction 1. In addition, with  $FAH_4$ , fixation of  $C^{14}$ -formate could be demonstrated in the absence of the acceptor, IRMP. This was interpreted as evidence for reaction 3. Dihydrofolic acid in this system also was able to stimulate formate fixation in the absence of IRMP, and this fixation was increased by DPNH. The finding of an interaction between DPNH and dihydrofolic acid is provocative, since it may suggest a role for the latter in electron transport. There was only slight  $C^{14}$ -formate fixation when folic acid was employed, and this was not enhanced by DPNH or



ascorbic acid. Under other conditions folic acid appears to be utilized (22).

With this evidence for reaction 3 and the means of labeling the cofactor with  $C^{14}$ , it was possible to test reaction 2, the ability of the formylated active cofactor to donate to IRMP to give IMP (Table 6).

TABLE 6  
FORMYL TRANSFER FROM COFACTOR TO IRMP

Total counts fixed as $FAH_4 \cdot CHO$ after 30 min.	13,100
Formate pool and IRMP-5 added and incubated additional 10 min.	
Isolated Inosinic acid	2960 counts/ $\mu M$ .
Formate Pool	250 counts/ $\mu M$ .

Conditions: 80 mg. Dowex-treated enzyme, 2.7  $\mu M$ .  $FAH_2$ , 0.4  $\mu M$ . DPNH, 10  $\mu M$ . ATP, 28  $\mu M$ . phosphoglycerate, 34  $\mu M$ . DL-homocysteine, 30  $\mu M$ .  $KHCO_3$ , 12.8  $\mu M$ .  $MgCl_2$ , 1.67  $\mu M$ .  $C^{14}$ -formate 51,000 counts/ $\mu M$ . 2 mg. muscle enzyme, vol. 2.69 ml. After 30 minutes, added 300  $\mu M$ . formate and 1.26  $\mu M$ . IRMP-5 and incubated 10 min. Temp. 38° C. Vol. 3.09 ml. The reaction was stopped by boiling 2 minutes.

$FAH_4$  (in this experiment  $FAH_2 + DPNH$ ) and  $C^{14}$ -formate were incubated for 30 minutes (reaction 3), at which time unlabeled formate (to dilute the  $C^{14}$ -formate 200-fold) plus IRMP were added and the mixture was incubated for 10 additional minutes (reaction 2). IMP and formate were isolated at the end of the reaction period. Since the IMP isolated had a specific activity 10 times that of the formate pool, a direct transfer of  $C^{14}$  from the cofactor synthesized in the preincubation to IRMP must have occurred.

Although a requirement for ATP in the overall reaction 1 was demonstrated, it was possible to show that it was not required in reaction 2, the transfer of the formyl group from the cofactor to IRMP. Substrate levels of  $FAH_4CHO$  were synthesized by the enzymatic reaction between CF and ATP. The ATP was then destroyed with apyrase, and IRMP was added and further incubated. Under these conditions IRMP disappeared, a change providing evidence for the occurrence of the transformylation reaction 2 in the absence of ATP. However, ATP is involved in the reaction of formate with the deformylated cofactor (reaction 3). It is also involved, as

previously mentioned, in the conversion of citrovorum factor to the active cofactor. Thus, the evidence presented indicates that the function of the cofactor is to accept and transfer formyl groups.

The second problem concerns the actual structure of the active cofactor, and determination of this was dependent upon its isolation.

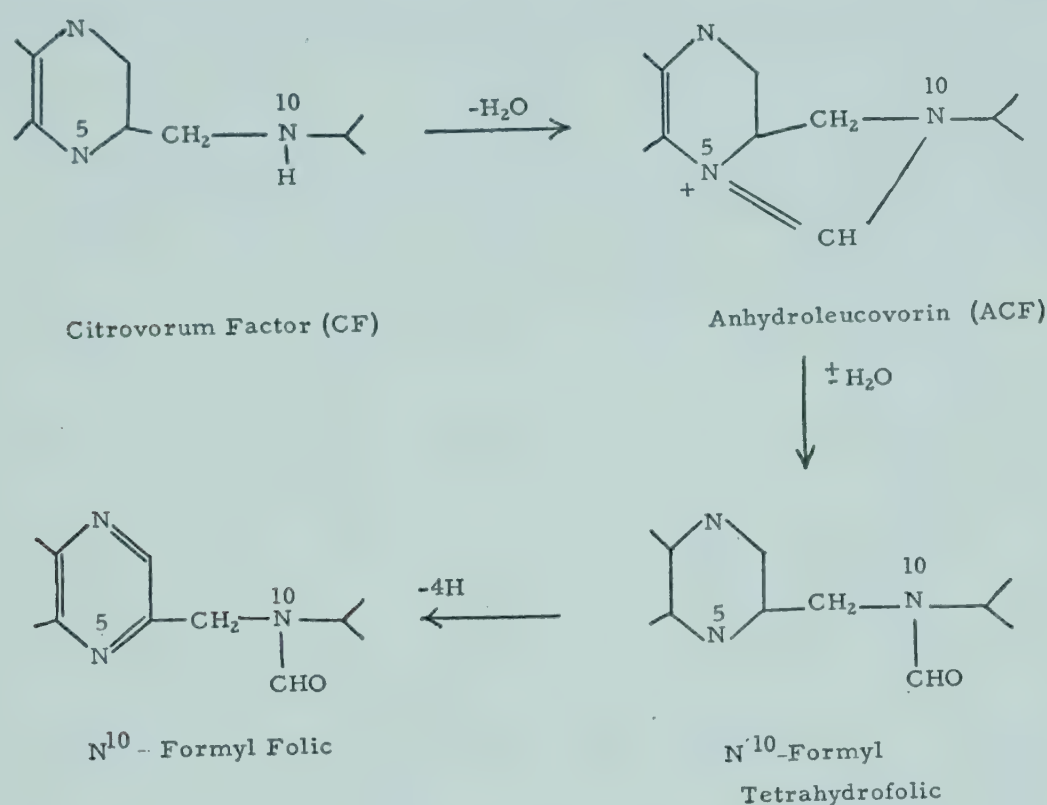


FIG. 3. Relationships of certain acid derivatives (Ref. 8).

When  $FAH_4$  (or  $FAH_2 + DPN$ ),  $C^{14}$ -formate, and ATP were incubated with the Dowex-1 treated enzyme, and the heat-denatured filtrate of the reaction was chromatographed on paper in a neutral solvent, several blue fluorescent bands appeared, one of which was radioactive. When this was eluted and used as the one-carbon substrate in the conversion of IRMP to IMP (reaction 2), IRMP-5 disappeared and no ATP was required. However, on further purification by paper chromatography with neutral solvents the biological activity was lost. The compound isolated appeared to be identical with synthetic N<sup>10</sup>-formyl folic acid, judged by spectrum, loss of the  $C^{14}$ -formyl group, and formation of folic acid in alkali, microbiological activity for *S. faecalis* R., and  $R_f$  in five different solvent systems.

Since citrovorum factor, which has its formyl group on the N<sup>5</sup> position, was converted by an enzymatic reaction with ATP to a



compound which on purification broke down to N<sup>10</sup>-formyl folic acid (Fig. 3) and since N<sup>10</sup>-formyl folic acid was inactive as a formyl donor in this system, it was reasonable to suppose that in the conversion the bridge compound anhydroleucovorin (ACF) might be an intermediate. This compound can be prepared chemically (26, 8) and was tested in substrate quantities as a formyl donor to IRMP.

TABLE 7  
REACTION OF ANHYDROLEUCOVORIN AND IRMP

Additions	1	2	3	4
Enzyme	+	+	+	+
CF	0	+	0	0
ACF	0	0	+	+
IRMP	+	+	+	0
Total Diazo Units	190	179	32	15

Conditions: as in ref. 17, but with the indicated addition plus bicarbonate buffer.

Results (Table 7) indicate that ACF can react with IRMP. This reaction does not take place with the carboxamide riboside or with the free base. The specificity of this reaction, its enzymatic nature, and the chemical characteristics of ACF suggest that ACF is donating a formyl group to IRMP. However, it remains to be determined whether the principal product is IMP.

Whether ACF is produced by the incubation of CF (or FAH<sub>4</sub> plus formate) with ATP is not clear. The partially purified cofactor, which was made enzymatically and retained some biological activity, had a spectrum unlike ACF but resembling that of either dihydro- or tetrahydro-N<sup>10</sup>-formyl folic acid. Since the dihydro form is more stable, the isolated substance was more likely this compound. Although ACF reacts in the system, it cannot be stated at the present time whether it or tetrahydro-N<sup>10</sup>-formyl folic acid or dihydro-N<sup>10</sup>-formyl folic acid or some other closely related derivative is the active cofactor. The role of ATP in the conversion of CF to the active cofactor could be in the formation of a bridge structure in which one of the nitrogens must carry a positive charge.

The cofactor obtained either from the reaction of CF and ATP, or from FAH<sub>4</sub>, formate and ATP is unstable, and therefore is probably not the natural moderately stable cofactor present in boiled extract. Silverman and others (6, 35) have shown that the boiled extract cofactor is present in a bound form and may be liberated by enzymatic reactions. They find that on anaerobic incubation of boiled liver extract with liver homogenate, a compound is formed which possesses microbiological activity for *S. faecaelis* but not for *L. citrovorum*, and this compound on purification yielded N<sup>10</sup>-formyl folic

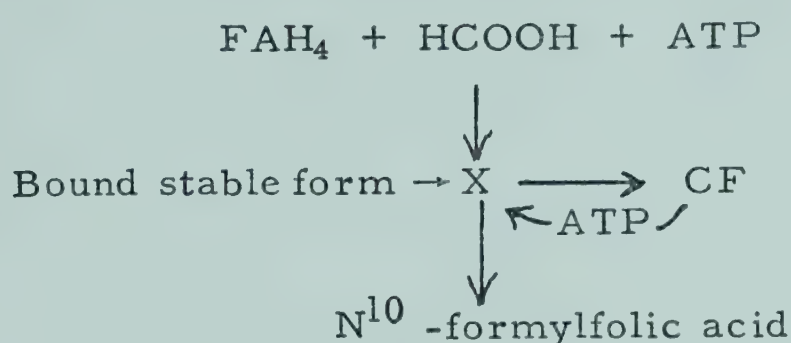


FIG. 4. Suggested interrelationships between stable cofactor form and folic acid derivatives.

acid. If the incubation was continued, citrovorum factor accumulated. However, N<sup>10</sup>-formyl folic acid was not converted to CF by the homogenate. The possible relationship between these findings and those presented appears in Fig. 4. Thus the product of the CF + ATP reaction, that of the FAH<sub>4</sub> + HCOOH + ATP reaction, and the product of the enzymatic release of the bound form in liver, may be a compound, possibly N<sup>10</sup>-formyl tetrahydrofolic acid, which can be degraded to N<sup>10</sup>-formyl folic acid.

The interrelationship of formate, the  $\beta$ -carbon of serine, and the 2-carbon of inosinic acid is illustrated in Fig. 5. Similar interconversions should apply to the carbon-8 of purine. Evidence for the reactions presented in this scheme is as follows. For the exchange reaction, serine  $\rightleftharpoons$  glycine + "C-1," Kisliuk and Sakami (22) and Blakley (2) have shown a requirement for tetrahydrofolic acid, and it has also been shown that in the presence of tetrahydrofolic acid and presumably DPNH, serine will donate its  $\beta$ -carbon to IRMP (18). In the glycine-serine exchange reaction, no ATP appears to be



required, while in purine synthesis from serine it is doubtful that it is involved. Kisliuk and Sakami (22) have also shown that tetrahydrofolic acid and ATP are involved in the synthesis of serine from glycine and  $C^{14}$ -formate. The cofactor requirement for the incorporation of  $C^{14}$ -formate into IRMP has been discussed. Finally, Flaks and Buchanan (11) have demonstrated a requirement for CF (in the absence of ATP) in the conversion of carbon-2 of inosinic

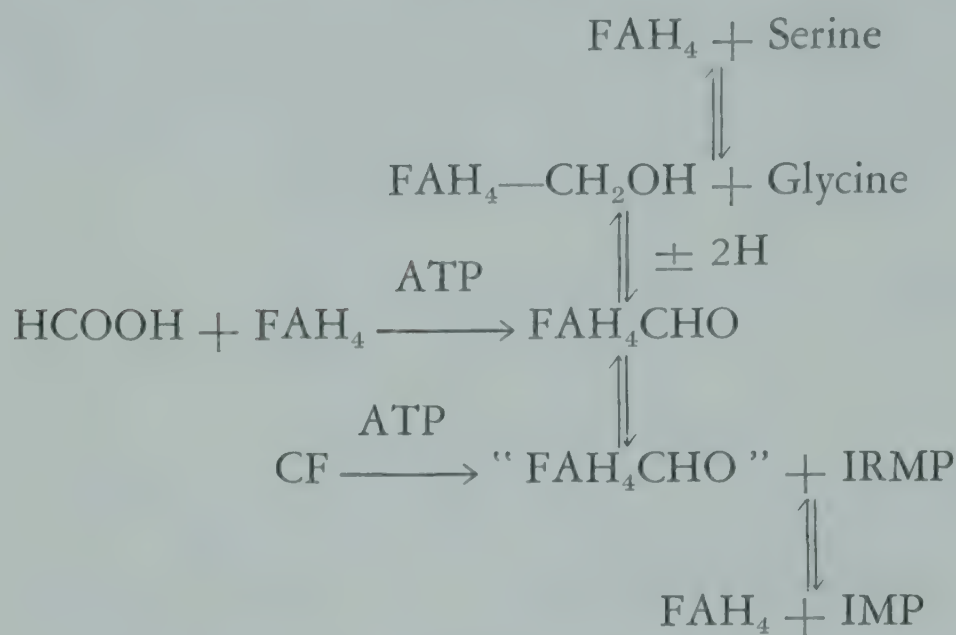


FIG. 5. Scheme for interrelationship of serine  $\beta$ -carbon and inosinic 2-carbon.

acid to the  $\beta$ -carbon of serine. Thus tetrahydrofolic acid derivatives appear to be involved in these reactions. The exact nature of these derivatives is not clear. The studies of Elwyn and Sprinson (10) with  $C^{14}$ - and deuterium-labeled formate in vivo showed that only 10 per cent of the deuterium was lost in the conversion to the  $\beta$ -carbon of serine, while 70 per cent was lost in the conversion to purine. If formate reacts initially with tetrahydrofolic acid in the presence of ATP, then the pathways to serine and purine must diverge in order to account for labilization of the deuterium only during purine synthesis. If this purine pathway involved ACF, such a labilization might occur due to the positive nitrogen or onium structure of this compound. Substantial evidence for or against such a scheme will depend upon isolation of the enzyme responsible for the interconversions, as well as characterization of the cofactors at different oxidation levels.

MECHANISM OF CO<sub>2</sub> FIXATION

The mechanism by which CO<sub>2</sub> is fixed into the purine ring is obscure in two respects. First, we do not know the structure of the intermediate to which CO<sub>2</sub> is added. Since radioactive carbon from CO<sub>2</sub> does not appear in the glycine ribotides (I, II) and their accumulation, in contrast to purines, does not depend upon CO<sub>2</sub>, we must assume that CO<sub>2</sub> is fixed at a later step. The recent experiments with biotin-deficient yeast in which CO<sub>2</sub> fixation might be impaired (5) and with purine-requiring *E. coli* (24) suggest that an amino imidazole ribotide (III) may be the compound with which CO<sub>2</sub> or a derivative condenses.

Secondly, it seems unlikely that CO<sub>2</sub> per se is fixed into the purine precursor. More probable is its fixation into some compound which then condenses with the intermediate. An obvious possibility is one of the members of the tricarboxylic acid cycle, and the recent work on the condensation of a succinyl derivative with glycine (33) suggested the possibility of this reaction as a mechanism for CO<sub>2</sub> fixation into purine. However, this appears to be ruled out. When C<sup>14</sup>-1,4-succinate incorporation into purines in a pigeon liver homogenate was compared to CO<sub>2</sub> incorporation in the absence and presence of malonate (12) (Table 8), it was clear that succinate

TABLE 8

INCORPORATION OF C<sup>14</sup>O<sub>2</sub>, AND 1-4 C<sup>14</sup>-SUCCINATE INTO  
PURINES IN PIGEON LIVER HOMOGENATES

Substrate	$\mu M.$ C <sup>14</sup> Fixed in Hx	Hx c.p.m./ $\mu M.$	Final CO <sub>2</sub> c.p.m./ $\mu M.$
C <sup>14</sup> O <sub>2</sub>	0.76	710	2490
C <sup>14</sup> O <sub>2</sub> + succinate	1.03	1005	1810
C <sup>14</sup> O <sub>2</sub> + succinate + malonate	0.59	708	2550
CO <sub>2</sub> + C <sup>14</sup> -succinate	0.11	20	71
CO <sub>2</sub> + C <sup>14</sup> -succinate + malonate	0.005	1	6



was one-tenth as effective a precursor as  $\text{CO}_2$ . When an unlabeled pool of the complementary substrate was employed as indicated, the inhibitory effect of malonate on  $\text{C}^{14}$ -succinate incorporation was far greater than on  $\text{C}^{14}\text{O}_2$  incorporation, a relation which suggested that succinate must be converted at least to fumarate before incorporation. The earliest work on purine synthesis in vitro suggested the possibility that oxaloacetate was involved (28). Dilution experiments with unlabeled oxaloacetate and labeled  $\text{CO}_2$  (12) suggest that oxaloacetate may be the immediate precursor of C-6, but direct experiments with labeled oxaloacetate must be done to prove this hypothesis. Citrulline and arginine are unlikely sources of the carbon-6 atom, since neither showed any effect in dilution experiments (12).

It is apparent that an intimate knowledge of the pathway of biosynthesis of the purine ring is lacking for most of the steps. However, there are a few signposts on this pathway which may help in the further elucidation of the problem.

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# EVIDENCE FOR A SPECIFIC ALANINE-HYDROXYPYRUVIC TRANSAMINASE \*

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IT HAS BEEN found that the administration of  $C^{14}$ -carboxyl-labeled glyceric acid to rats gave rise to a significant amount of radioactivity in the serine isolated from the tissue proteins (1). Furthermore, degradation studies demonstrated that the isotope resided predominantly in the carboxyl carbon of the serine molecule. These results suggested a possible metabolic relationship between glyceric acid, hydroxypyruvate, and serine. On this basis it appeared worthwhile to investigate the possible participation of hydroxypyruvate as an intermediate in the conversion of glyceric acid to serine via transamination reactions. In the present report evidence will be presented for the formation of serine by means of an alanine-hydroxypyruvic transaminase.

Quantitative determinations of serine were made by measuring the ammonia liberated after oxidation with periodate (2). Control experiments with hydroxypyruvate alone or alanine alone gave no observable serine formation. The presence of serine and alanine in the reaction mixtures was confirmed qualitatively by paper chromatography.

A survey of a variety of tissues from different animals is summarized in Table 1. The results indicate that only liver and kidney have significant activity. A partial purification of the enzyme from dog liver has been achieved by heat treatment of an acetone powder extract followed by low temperature ethanol fractionation. On the basis of protein content, the partially purified preparation has some fifty times the activity of a whole liver homogenate.

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TABLE 1  
TRANSAMINASE ACTIVITY OF VARIOUS TISSUES IN THE PRESENCE OF  
HYDROXYPYRUVIC ACID AND L-ALANINE

Tissue	Total serine formed/hr. $\mu$ M.	$\mu$ M. serine/mg. protein/hr.
Extracts * of acetone powder of:		
dog liver	25.6	0.6
" kidney	11.3	0.4
" heart	0.7	0.04
" brain	0.3	0.01
" spleen	0	0
rabbit liver	11.5	0.3
" kidney	1.7	0.04
" brain	0	0
beef liver	2.7	0.08
Homogenates † of:		
rat liver	2.2	
" kidney	2.8	
" heart	0.3	
" brain	0.4	

\* 1 g. of acetone powder extracted with 10 ml. of 0.01 M.  $\text{PO}_4$ , pH 7.4, for 20 minutes at 0° C. Supernatant dialyzed vs. 0.01 M.  $\text{PO}_4$  for 10 hours.

† 250 mg. of tissue (wet weight) per flask.

The reaction systems contained 40  $\mu$ M. hydroxypyruvic acid, 40  $\mu$ M. L-alanine, and 30  $\gamma$  pyridoxal phosphate in a volume of 3 ml. Incubation time, 1 hour; 0.01 M.  $\text{PO}_4$ , pH 7.4.

TABLE 2

Reactants		Total $\alpha$ -keto acids reduced by lactic dehydrogenase ( $\mu$ M.)		Serine ( $\mu$ M.)		Alanine * ( $\mu$ M.)
Amino acid	$\alpha$ -Keto acid	0 time	1 hour	0 time	1 hour	1 hour
L-Alanine	Hydroxypyruvic	24.9	24.0	0	15.7	
L-Serine	Pyruvic	23.9	23.8	23.7	18.0	5.8
L-Serine	—	—	—	23.7	23.8	—

\* Measured by serine disappearance.

The reaction systems contained 25  $\mu$ M. of amino acid, 25  $\mu$ M. of  $\alpha$ -keto acid, 2.1 mg. of enzyme preparation, 30  $\gamma$  of pyridoxal phosphate in a volume of 3 ml. Incubation time, 1 hour; 0.01 M.  $\text{PO}_4$ , pH 7.4.



Convincing evidence for transamination reactions requires the demonstration of a stoichiometric balance of reactants and products in accordance with the proposed reaction. Preliminary balance studies for this system are shown in Table 2. Meister (3) has reported that the rate of reduction of hydroxypyruvate by reduced diphosphopyridine nucleotide in the presence of muscle lactic dehydrogenase is as rapid as the rate of reduction of pyruvate. If the reaction between hydroxypyruvate and alanine is one of transamination, equimolar quantities of pyruvate and serine should be formed. Therefore the total  $\alpha$ -keto acids reducible by muscle lactic dehydrogenase should remain constant during the course of the reaction. This was the observed result when the reaction was approached from either direction. The concurrent formation or disappearance of serine was noted in these systems as indicated.

Pyridoxal phosphate has been demonstrated as the coenzyme required for various transamination systems (4-6). This compound was found to stimulate the formation of serine from hydroxypyruvate by approximately 35 per cent with the purified enzyme, indicating a partial resolution during purification. These observations clearly indicate that the reaction observed is one of transamination.

A number of amino acids were investigated as potential amino group donors (Table 3). Of those tested, alanine was the only one to show a significant activity. The slight formation of serine observed with glutamic acid remains unexplained. In cruder preparations the activity of glutamic acid was equivalent to that of alanine. However, following dialysis only alanine was fully active. Furthermore, it was necessary to add catalytic amounts of pyruvic acid to the dialyzed preparation to restore glutamic acid activity. These results suggest that the original activity of glutamic acid was due to a coupled transamination with glutamic-pyruvic transaminase. It is, however, still possible that a hydroxypyruvic-glutamic acid transaminase exists, since neither the crude dialyzed preparations nor the ethanol-fractionated enzyme completely loses activity for glutamic acid. In relation to this, Meister and coworkers (7) have reported the formation of serine from hydroxypyruvate and glutamine by an enzyme purified from rat liver.



TABLE 3

TRANSAMINATION BETWEEN AMINO ACIDS AND HYDROXYPYRUVIC ACID  
BY PARTIALLY PURIFIED ENZYME

Amino acid	Total serine formed/hr. $\mu$ M.
L-Alanine	16.8
L-Glutamic acid	0.7
L-Aspartic acid	0
L-Phenylalanine	0
L-Leucine	0
L-Valine	0
DL-Methionine	0

The reaction systems contained 40  $\mu$ M. of the L-form of the amino acid, 40  $\mu$ M. hydroxypyruvic acid, 2.2 mg. enzyme preparation, 30  $\gamma$  pyridoxal phosphate in a volume of 3 ml. Incubation time, 1 hour; 0.01 M.  $\text{PO}_4$ , pH 7.4.

Due to the relatively high activity of the hydroxypyruvic-alanine transaminase in liver and kidney, it appears that it represents a major pathway of serine metabolism in these tissues. A tentative scheme relating the reaction discussed here to the metabolic origin of hydroxypyruvate from the glycolytic scheme is shown in Fig. 1. Existing evidence suggests several possible routes for the conversion of D-glyceric acid into hydroxypyruvate. The reduction product of the latter compound by muscle lactic dehydrogenase and reduced diphosphopyridine nucleotide has recently been identified as L-glyceric acid (8). However, in view of the fact that a racemase for D- and L-lactic acids has been reported (9), it is conceivable that this enzyme, or a similar one, could catalyze an analogous reaction between D- and L-glyceric acids. Alternatively, Baker (10) has presented evidence for the occurrence in mammalian tissue of both D- and L-specific  $\alpha$ -hydroxy acid oxidases. Although D-glyceric acid apparently was not investigated, its oxidation to hydroxypyruvate by a D- $\alpha$ -hydroxy acid oxidase would provide a more direct pathway for hydroxypyruvate synthesis. On the other hand, the direct formation of hydroxypyruvate from intermediates in the "oxidative" or non-glycolytic pathway of carbohydrate metabolism should not be excluded.



Also included in the proposed scheme is a possible route for phosphoserine formation. In the event that D-3-phosphoglyceric acid is oxidized to phosphohydroxypyruvate, the latter compound could,

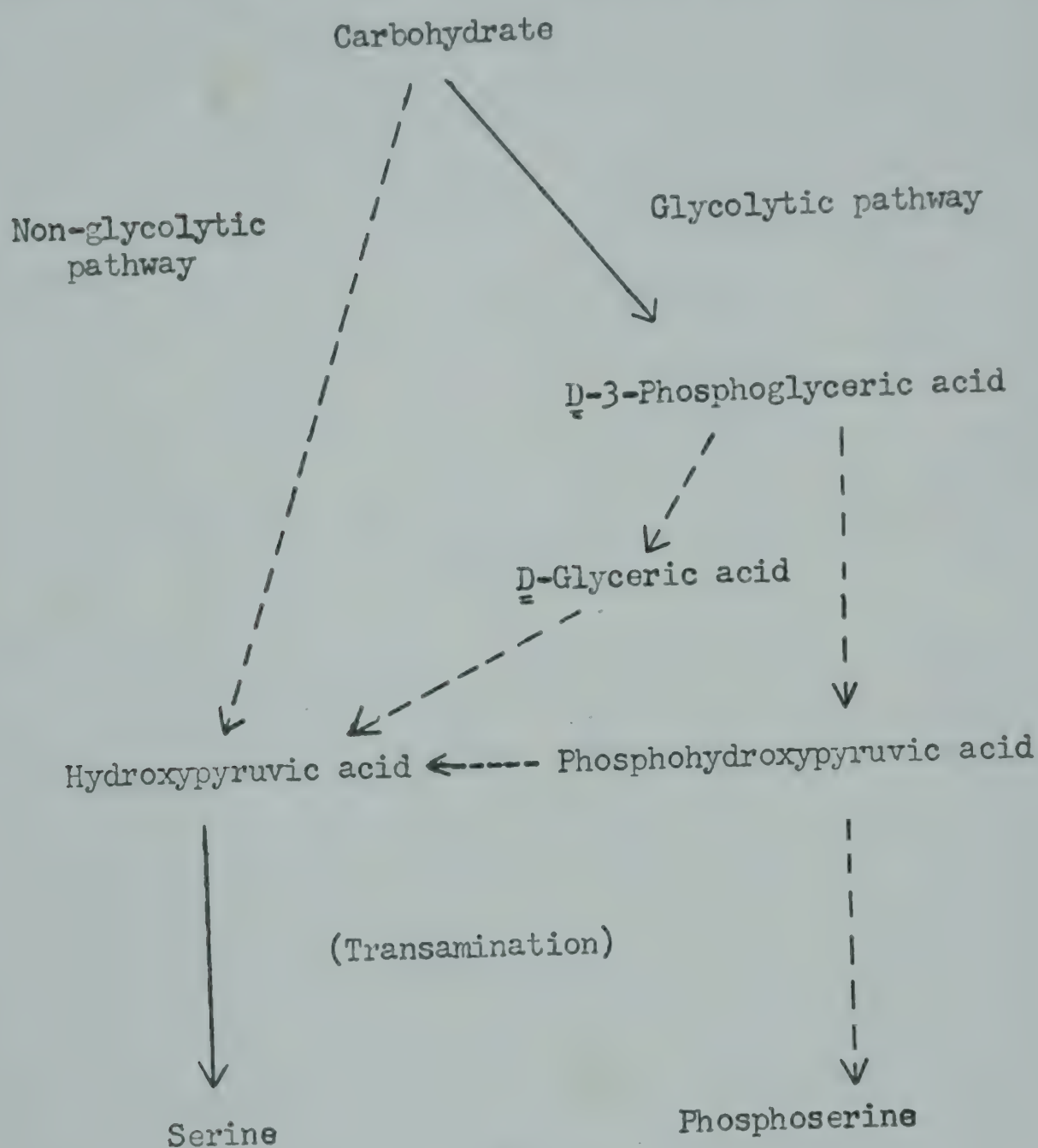


FIG. 1.

by transamination, yield phosphoserine directly. The significance of these systems in the metabolism of serine and phosphoserine is being further explored.

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## DISCUSSION

DR. WOOD: We will open the meeting for discussion. I think you all will agree that it has been a very exciting afternoon, with new cycles being postulated and evidence shown elucidating the role of tetrahydrofolic acid. How the citrovorum factor is active in carrying the formyl group, has been puzzling people for a number of years. There have been suggestions as to different methods of oxidizing glycine through glyoxylic acid, and the role of sarcosine in synthesis. All of these mechanisms are linked together in a way in which we can't even visualize, in spite of the brilliant work that has been presented. I think there are probably many ideas in this group which are just waiting to be popped. Some people have approached me, so I'll let them start the discussion.

DR. B. WRIGHT: I would like to present some preliminary data on another system in which serine-glycine interrelationships can be studied. This system involves the net production of glycine from serine in the presence of cell-free extracts of *Clostridium HF*, an unidentified strain isolated by Thressa Stadtman. The glycine formed is assayed by its conversion to formaldehyde which is determined by a colorimetric method.

The conversion of serine to glycine by dialyzed extracts involves a requirement for DPN,  $Mn^{++}$ , and pyridoxal phosphate. Folic acid and citrovorum factor do not stimulate the system significantly. Treatment with Dowex 1 completely inactivates the system so that it can no longer respond to DPN, Mn, and pyridoxal phosphate. Striking reactivation is observed, however, in the presence of these three cofactors and catalytic levels of a boiled extract



of *Clostridium cylindrosporum*. Preliminary experiments indicate that the boiled extract activity is not due to the presence of folic acid, citrovorum factor, or tetrahydrofolic acid. Another more active cofactor may be required in this system.

DR. GUNSALUS: I should like to return to Dr. Weinhouse's presentation, particularly to his reference to the experiments of Campbell, Smith, and Eagles, which were reported a year ago on a new cleavage reaction of tricarboxylic acids. Several investigators, particularly Krebs, have referred to the likelihood that the citric acid cycle is at least as important as a source of synthetic intermediates as for coupled energy transfer during complete oxidation. I should like to add some new data on the synthetic intermediate side which Mr. Smith, now in our laboratory, has added on the system which he and Campbell were studying. I became involved in this problem only because of being a scientific grandfather. Campbell is at the University of British Columbia, which does not award Ph. D.'s in microbiology, and Smith is at the University of Illinois. The initial experiments showed the formation of glyoxylate and of succinate from citrate cis-aconitate. Smith has now shown that extracts of this pseudomonad freed of aconitase activity cleaves only isocitrate, and not citrate or cis-aconitate, to yield glyoxylate and succinate. Thus we refer to the system, possibly one enzyme, as "isocitritase" in comparison to the citrate cleavage system which we previously described in *Streptococcus faecalis* and *Escherichia coli* yielding oxaloacetate and acetate which was termed "citridesmolase" or "citritase." Both of these would appear to be aldolasetype cleavages. The isocitritase has been purified several-fold and shown to require for activation a divalent metal, of which magnesium is the most active, and a mercapto compound, of which cysteine and glutathione are approximately of equal activity. Coenzyme A is not required. Since Campbell and Smith had indicated in their initial communication that the system was reversible, we have examined this possibility but so far with the partially purified extracts satisfactory evidence for reversibility has not been obtained. Succinate, one of the products of the cleavage, is inhibitory to the forward reaction, possibly indicating displacement of the equilibrium or affinity for the enzyme. Further data will be required to clarify this point. These data, I believe, indicate the first enzymatic reaction in the tricarboxylic cycle affording new synthetic intermediates. Glyoxylate, as pointed out by Dr. Weinhouse, is the most likely precursor of glycine and may well serve as an immediate precursor of the metabolically-active  $C_1$  compound now under intensive study.

One could point out a number of interesting possibilities arising from the two asymmetric carbons in isocitric acid in terms of the labeling to be expected in oxidative  $C_1$  vs. tricarboxylic acid cycles. But the hour, I believe, precludes the inflicting of these considerations, regardless of their importance, upon the meeting.



DR. UDENFRIEND: I would like to ask Dr. Mackenzie how specific is that oxidative demethylation. Does it work only on monomethyl and dimethyl glycine, or are there other compounds which are acted upon? Is it found only in mitochondria?

DR. MACKENZIE: It won't work on monomethylaminoethanol, dimethylaminoethanol or on choline or betaine or on methyl amine or dimethyl amine. I have not tried some of the compounds which you mentioned. What N-methyl compounds are you most interested in?

DR. UDENFRIEND: Will the mitochondria system demethylate butter yellow?

DR. MACKENZIE: Such a system will, I think, demethylate butter yellow. In 1949 Boissoness, Turner and du Vigneaud (*J. Biol. Chem.* 180, 1053, 1949) synthesized methyl labeled butter yellow and showed that it was oxidized to  $C^{14}O_2$  by the intact rat. Kensler and I succeeded in isolating radioformaldehyde as a product of its oxidation by liver slices. This was published in the Transactions of the Fourth Macy Conference on Biological Antioxidants (Ed. C. G. Mackenzie, Josiah Macy, Jr. Foundation, New York, 1950).

DR. HANDLER: Dr. Ryoza Hirohata in Japan has described a flavoprotein from liver which oxidizes the  $\alpha$ -N-methyl derivatives of most of the common L-amino acids to the corresponding amino acids and formaldehyde. The prosthetic group appears to be FAD.

DR. MACKENZIE: In whole homogenates some of the compounds that I have mentioned like dimethylaminoethanol are oxidized, but in mitochondria they are not, and they are not even inhibitors of the oxidation of sarcosine.

DR. COHEN: I would just like to mention that Dr. Tung in our laboratory demonstrated that N-methyl tryptophan was oxidized to form formaldehyde and formic acid in liver and kidney homogenates.

DR. KAPLAN: I was wondering, Dr. Mackenzie, why in your experiments free formaldehyde does not serve as a precursor for serine. This would be in contrast to Sakami's experiments. I believe his system does incorporate formaldehyde. Since you used mitochondria, is the formaldehyde activation system in the soluble components? I wonder if you have any comments on that.

DR. MACKENZIE: I think Dr. Sakami anticipated this in part by supposing that perhaps the oxidation product of sarcosine or dimethyl glycine is able to employ a built-in tetrahydryl folic acid in mitochondria, if I got what he said, which was perhaps not loud enough to reach the back of this room. This is a possibility which we also have anticipated. Now, in folic acid deficient rats—that is, in rats not getting folic acid or  $B_{12}$  in their diet—the growth is quite normal, but the mitochondria from such rats do not



oxidize sarcosine at the normal rate. It is oxidized at about 60 per cent of the normal rate. However, the ratio of formaldehyde to serine formed is unchanged. There is no indication of depressed serine synthesis. It would appear that one of these vitamins, either directly or indirectly, is involved perhaps in the dehydrogenation reaction itself. It could be that the same coenzyme existing in juxtaposition between two enzymes could pull off the hydrogens from sarcosine or dimethylglycine and grab the one carbon compound at the same time, subsequently transferring it to glycine. I don't like to speak for you, Dr. Sakami, but isn't that also the essence of your comments? Maybe the H atoms removed from the methyl glycine pass the potential required for the formation of tetra-hydrofolic acid in mitochondria.

DR. SAKAMI: Yes. The activation of formaldehyde appears to occur more rapidly in the soluble fraction of rat liver than in the particles. Washed rat liver particles incorporate formaldehyde- $C^{14}$  into serine- $\beta$ -carbon (after the addition of tetrahydrofolic acid) much more slowly than the soluble fraction of rat liver homogenate, even though the introduction of the active  $C_1$  unit into serine appears to be considerably more rapid.

The incorporation of sarcosine methyl carbon into serine in rat liver mitochondria is a rapid process which may not involve formaldehyde *per se* as an intermediate. Mr. Deodhar and I have considered the possibility that folic acid or dihydrofolic acid is involved as a cofactor in sarcosine methyl oxidation. The reduced cofactor would then remove the oxidized methyl group of sarcosine as hydroxymethyldihydrofolic acid or hydroxymethyl-tetrahydrofolic acid, respectively, and transfer it to the glycine- $\alpha$ -carbon. We have not yet succeeded in obtaining experimental evidence for this hypothesis. The addition of substrate amounts of folic acid or dihydrofolic acid to liver particles did not increase the anaerobic oxidation of sarcosine.

DR. MACKENZIE: That has been our experience. Added folic acid does not increase the rate of oxidation of sarcosine by mitochondria. Adding folic acid does nothing but disrupt some part of this conversion of sarcosine to serine. As a matter of fact almost anything you put in will screw it up in one way or another, but this is no proof that the added coenzyme isn't built in the mitochondria to begin with. Indeed FAD appears to be built in, yet added FAD oxidizes formaldehyde and active formaldehyde.

DR. HANDLER: Perhaps it is naive, but I should like to point out that the oxidation of sarcosine to glycine and formaldehyde must involve either the  $-N=CH_2$  or  $-N-\underset{H}{CH_2}OH$  derivatives, or both, as intermediates and it is conceivable that one of these serves as the active  $C_1$  donor derived from sarcosine.

DR. DU VIGNEAUD: I would like to inquire if you have made any studies in your *in vitro* enzyme system on glycoamine and methyl thioglycolic acid.



These of course are the compounds that would be left after one methyl group has been removed from betaine and dimethylthetin, respectively. The corresponding methyl-thiopropionic acid which might arise from dimethyl-propiothetin is also of interest.

DR. MACKENZIE: The same enzyme system does handle it in a very interesting way. Methyl thioglycolic acid is an excellent inhibitor for sarcosine oxidation, and you get no formaldehyde formed from it in this system. It's almost as good as methoxyacetic acid as an inhibitor. Both are competitive inhibitors and are not touched themselves to any great extent. There may be some exchange of their methyl hydrogens in a monovalent reversible oxidation but no demethylation.

DR. T. C. STADTMAN: Is thioglycolate also an inhibitor of sarcosine oxidation? In the *Clostridium HF* system the synthesis of doubly-labeled acetic acid from labeled formate is completely inhibited by thioglycolate but not by sodium sulfide. Since one of the known sources of acetate in the system is glycine, one wonders if thioglycolate may be acting as a competitive inhibitor of an intermediate between glycine and acetate, for example.

DR. MACKENZIE: No. We haven't tried that compound, but would you be interested in cysteine or homocysteine? We know that these compounds gobble up the formaldehyde and do not reduce the serine synthesis. Could I put this on the board? I was asked to get through in a given amount of time and had to leave something out. If one takes 10 micromols of sarcosine, you get from it 5 micromols of formaldehyde and 5 micromols of serine. If you add cysteine, you eliminate formaldehyde accumulation, but nothing happens to serine synthesis—that is, you still get 5 micromols of serine. Now, what happens here is that formaldehyde reacts with cysteine and is oxidized at a rapid rate, and we have very good evidence that it is oxidized through a thiazoladinecarboxylic acid. If you put cysteine in mitochondria alone you have an induction period, and then you get oxidation occurring. The final oxygen uptake corresponds to 1 microatom of oxygen per micromol of substrate. Now, if you add formaldehyde you get no induction period, and the oxygen consumption goes rapidly to the same end point, and you can demonstrate that thiazoladine carboxylic acid is formed; and if you put in thiazoladinecarboxylic acid itself the rate of oxidation is somewhat faster than is obtained with cysteine plus formaldehyde. I would like to have a chance maybe to explore these reactions in this system and thiazoladine oxidase from mitochondria for a year, if anybody doesn't mind.

DR. CANTONI: So long as methyl thioglycolic acid and methyl thiopropionic acid have come up, I would like to tell of an experiment which Dr. Anderson and I have started in an attempt to get at the question of the



stereoisomerism of the onium compounds. We incubated  $C^{13}H_3-\overset{+}{S}-CH_2-COOH$  and unlabelled dimethylthetin with a highly purified preparation of homocysteine methyltransferase, the enzyme which transfers the methyl group of dimethylthetin to homocysteine to form methionine. Our hope was that the dimethylthetin would methylate the labelled methyl thioglycolic acid to form dimethylthetin in which one of the methyl groups is not labelled.

If 
$$\begin{array}{c} CH_3 \\ \diagdown \\ \overset{+}{S} \\ \diagup \\ C^{13}H_3 \end{array} - CH_2 - COOH$$
 is formed it becomes possible to test whether

the two methyl groups are equivalent or if one is preferred in transmethylation reactions. To this end we had hoped to isolate the thetin and use the partially labelled thetin as a methyl donor for methionine synthesis. If the methyl group in methionine which is derived from this partially labelled thetin is not labelled this would provide good evidence that there is a stereoisomerism involved in the transfer of the methyl group from onium compounds. Up until the day before I left, the experiments which we had started weren't in good shape and had given negative results. In other words, we had not been able to show that dimethyl thetin or dimethyl propiothetin methylates methyl thioglycolic acid or methyl thiopropionic acid. One catch in the experiments which we have done is the fact that the enzyme may well be a sulfhydryl enzyme, as are all other methyltransferases. The experiments which had been done before I left were done without the addition of the sulfhydryl groups. We plan to do experiments with glutathione and cysteine. However, should the  $-SH$  requirements be specific for homocysteine, then there would be no way of getting at this particular problem.

DR. SPRINSON: Dr. Weinhouse has mentioned an experiment in which ribose-1- $C^{14}$  was administered together with benzoate to a rat and activity found in the excreted hippurate. A similar result was obtained by Dr. Weissbach in our laboratory. However, degradation of the glycine showed that the activity was not predominantly in one atom, approximately 60 per cent being in the carboxyl group. By analogy with the conversion of ethanolamine-2- $C^{14}$  ( $C^{14}H_2NH_2 \cdot CH_2OH$ ) to glycine-1- $C^{14}$  (Weissbach and Sprinson, *J. Biol. Chem.* 203, 1031 (1953)) one would expect the "active glycolaldehyde" derived from ribose-1- $C^{14}$  to yield primarily glycine-2- $C^{14}$ . The high activity found in the carboxyl group suggests that indirect pathways may have made a significant contribution to the utilization of pentose for glycine formation. Moreover, the extent of conversion of pentose to glycine in the rat, unlike that reported here by Weissbach and Horecker in spinach extracts, was quite low. Certainly the conversion of ribose-1- $C^{14}$  to methyl-labeled acetate which we observed later (Sprinson and Weliky, *Federation Proc.* 13, 302 (1954)) was much more extensive.



In view of Dr. Sallach's report of a transamination between alanine and hydroxypyruvate to yield serine it is worth recalling that *in vivo* transamination from serine is quite limited (Elwyn and Sprinson, *J. Biol. Chem.* 207, 459 (1954)).

DR. MEHLER: I would like to ask some of the purine people whether some of the effects of ATP might be due to the conversion of DPN to TPN. Has TPN been tested in the systems which you have studied, and is it effective?

DR. SAKAMI: This is a possibility. We have no evidence on this point.

DR. SIMMONDS: I would like to add to the now increasing evidence that the carbon atoms of glycine may arise from sources other than serine. In our laboratory we have just finished experiments using a mutant of *E. coli* (strain K12) which has an absolute requirement for serine and cannot use glycine in place of serine. This mutant was grown on uniformly labelled glucose- $C^{14}$  in the presence of unlabelled L-serine. The serine isolated from the proteins of the organisms was completely cold, indicating that all of the serine in the proteins came exclusively from serine added to the medium. One third of the bacterial glycine is derived from the carbon supplied as isotopic glucose—in other words, at least one-third of the glycine has come by a pathway in which serine did not serve as a normal intermediate. To link this afternoon's discussion with this morning's, I might say that the cystine isolated from the proteins of *E. coli* was derived at least 98 per cent from the serine supplied in the medium and only about 2 per cent from the glucose carbon.

DR. STEKOL: We reported that totally-labelled glucose, fed to the intact rat, yields labelled serine, choline, creatine, and the methyl group of methionine. The extent of the labelling under the same conditions is quite good, considering the pool, if you like, into which the dietary glucose enters. In addition, formaldehyde  $C^{14}$  gives rise to glycine in the intact rat which has been isolated as hippuric acid.

DR. DAVIS: I should like to add something to Dr. Simmond's remarks. The many reactions involving serine or glycine that have been presented today give one the impression that there are innumerable ways of getting to and from these two compounds. Now these reactions, like any others that a cell can perform, are all interesting. But it seems to me particularly interesting to ask the following question: of the various reactions that can lead to the synthesis of a cell constituent, which ones constitute its *normal* biosynthetic route? (It seems useful to define this normal route as the one from a general carbon source, such as glucose.) Since it is not self-evident that there must be only one normal route, it should be pointed out that whenever a block in a single reaction causes the appearance of a growth requirement, one can pretty safely conclude that that reaction is part of an obligatory



pathway—i. e., that that species has no alternative mechanism, bypassing the blocked reaction, for forming the required compound from a general carbon source at an adequate rate. Most of the specific, terminal pathways of amino acid biosynthesis appear to be obligatory in this sense, as shown by the fact that auxotrophic mutants have been isolated for most of the amino acids.

The existence of mutants that respond to either serine or glycine seems therefore to imply that for this pair of compounds, too, there is only one significant normal biosynthetic pathway, at least in microbial cells. Without asking that that pathway be described in detail, one might nevertheless ask, on the basis of the information already available, whether serine or glycine comes first on that path. The existence of the kind of mutant mentioned by Dr. Simmonds, which responds to serine but not to glycine, suggests that glycine comes first.

DR. GUNSALUS: The bacterial transaminase data, I believe, would favor Dr. Davis' view that in *E. coli* glycine is synthesized directly. That is, there is a glycine but not a serine transaminase, as indicated by Feldman's data. This observation is the one which led us to look for biological systems forming glyoxylate rather than hydroxypyruvate as the most likely route to glycine and serine.

DR. R. B. ROBERTS: I would like to answer Dr. Davis in part. I think there are several "normal" routes of glycine-serine synthesis in wild type *E. coli*. When *E. coli* is grown on  $C^{14}$ -glucose, serine is derived directly from glucose and no  $CO_2$  gets into serine. When  $C^{12}$ -serine is added as a supplement, no  $C^{14}$  appears in either the serine or the glycine of the bacterial proteins. In contrast, the addition of  $C^{12}$ -glycine affects only the radioactivity of glycine; also exogenous  $C^{14}$ -glycine appears in the glycine but not in the serine of the protein. Furthermore, the 1-carbon of glucose appears in serine but not in glycine. In this case the predominant pathway is from serine to glycine.

However, there is also another pathway of glycine synthesis; when the cells are grown in the presence of  $C^{12}$ -glucose and  $C^{14}O_2$  some  $C^{14}$  appears in glycine but not in serine. Ten per cent of the glycine may be formed by this route which does not involve serine.

A third pathway comes into operation when threonine is added as a supplement.  $C^{14}$  from  $C^{14}$ -threonine appears in glycine but not in serine indicating a conversion of threonine to glycine.

A further complexity appears when fructose or acetate is used as the energy source. When  $C^{12}$ -glycine is added as a supplement to cells growing on  $C^{14}$ -fructose, the radioactivity of both glycine and serine is suppressed. This shows a conversion of exogenous glycine to serine which did not occur when glucose was used as the energy source. Perhaps the pathway from glucose to serine is a rather special and favorable one.



So there are several routes of glycine-serine synthesis which can operate in *E. coli* and some of them can operate simultaneously. The "normal" route then depends on what is considered to be the "normal" medium.

DR. DAVIS: Dr. Roberts and I had a further discussion after the meeting, and I think I am quoting him correctly in saying that we came to agreement that his data do not conflict with one of my two main points: namely, that on a given carbon source, such as glucose, there is only one major path to serine and glycine in *E. coli*.

As to whether on that path serine or glycine arises first, there is clearly a conflict between the isotopic results, which point to serine as the earlier product, and the nutritional observations on mutants, which point to glycine. Faced with the impressive isotopic evidence, I would concede that the interpretation I offered for the nutritional observations may well be wrong, these observations possibly involving as yet unknown complications. On the other hand, I do not believe that the isotopic observations reported have established the positive conclusion that the path proceeds from serine to glycine. In a sense, I think I phrased the original question incorrectly in asking whether glycine comes before serine or vice versa. If there is an obligatory normal path leading to glycine and serine, this does not imply that either compound is necessarily the normal precursor of the other; it is more logical to ask whether this path involves formation of serine by build-up of a 2-carbon precursor, or formation of glycine by breakdown of a 3-carbon precursor. Accordingly, the finding that added glycine gets incorporated into cellular glycine but not into cellular serine clearly excludes glycine as a normal precursor of serine (unless one invokes some rather fancy compartmentalization of the cell); but this finding does not exclude the possibility that serine might be normally synthesized from a conjugate of glycine, or from some 2-carbon precursor of glycine, whose endogenous formation was not effectively competed with by exogenous glycine.

DR. WOOD: I would like to ask a question of Dr. Shemin. I have never examined the isotope data closely on uric acid, but I wonder when alpha-labelled glycine is given is the number 6 position labelled highly enough so that you would consider the delta amino carbon to be a precursor of the 6-carbon of uric acid. If I understood correctly, that would be the carbon that would go to the 6th position. In the experiment that Sonne and others have done, I have never seen them interpret it as the alpha carbon group of glycine going to the 6th position.

DR. SHEMIN: Perhaps I haven't made myself clear, but the alpha carbon atom didn't go to the 6th position as such—it was  $\text{CO}_2$ . In a comparable experiment which we did using glycine-2- $\text{C}^{14}$  about 8 times as much  $\text{C}^{14}$  goes into  $\text{C}^2$  and into  $\text{C}^6$ . On the other hand, using delta amino levulinic acid the ratio is 1:1. What I was getting at is this, that if there is a series



of events in the succinate-glycine cycle which forms some compound on the oxidation level of  $\text{CO}_2$ —or let's say goes to some compound X which can be used as a formate derivative, and X can also go to Y, which can be used as a  $\text{CO}_2$  derivative, then this chain of events may explain the finding that the number 2 and 6 carbon atoms have the same activity. From these results it may be inferred that we have a compound which transfers a grouping equivalent to  $\text{CO}_2$  directly to the 6 position.

DR. BUCHANAN: Did you measure the  $\text{CO}_2$ -specific activity of either your animal or your in vitro system?

DR. SHEMIN: No.

DR. BUCHANAN: Then you will have to do that.

DR. SHEMIN: Oh, yes. This is very recent work.

DR. STEKOL: Dr. Shemin, what was the specific activity of the methyl group, wherever they were? Would you like to give us some figures on that?

DR. SHEMIN: As you noticed in that case, I just said that qualitatively they were labelled. We haven't got good comparable data at the moment with glycine. I have data only for the purine, serine, and methyl groups synthesized from delta amino levulinic acid,  $-5\text{-C}^{14}$ , and they were of the same order of magnitude.

DR. WOOD: I also wanted to ask Dr. Sakami a question for my own information. Didn't you run an experiment with labelled glyceric acid to see whether glyceric acid is a precursor for serine? You found that it was not a precursor, didn't you?

DR. SAKAMI: No. We have isolated choline after the administration of  $\beta\text{-C}^{14}$ -glyceric acid from rats. The methyl groups did not possess significant activity. The serine part of this experiment has not been completed.

DR. JAKOBY: Since Dr. Weissbach and Dr. Horecker have mentioned their work with a system which will fix  $\text{CO}_2$  with spinach extracts, I would like to add that Dr. Ochoa and I have been working on a very similar system. With an ammonium sulfate fraction from spinach leaves we have found an absolute requirement for ribose-5-phosphate,  $\text{Mg}^{++}$  and ATP for the incorporation of  $\text{CO}_2$ . In isolated experiments with a crystalline ATP preparation (Sigma) there was no incorporation of  $\text{CO}_2$  although a material rich in guanosine diphosphate and containing ATP did carry out the reaction. In experiments in which  $\text{C}^{14}\text{O}_2$  was employed most, if not all, of the  $\text{C}^{14}$  was found in phosphoglyceric acid which was obtained as the crystalline Ba salt.

DR. MAGASANIK: I think it is very dangerous to compare bacteria grown on different carbon sources and with different supplements, because the enzymes in these organisms are very much dependent on the nature of the medium in which they are grown. What may be a normal pathway under one set of conditions may be an unusual pathway in another condition.

## Part VI

### *METABOLISM OF AROMATIC AMINO ACIDS*





# BIOSYNTHESIS OF THE AROMATIC AMINO ACIDS

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BIOCHEMISTS HAVE LONG wondered about the mechanisms by which plants and microorganisms accomplish the feat of synthesizing various benzenoid compounds from non-aromatic source materials.

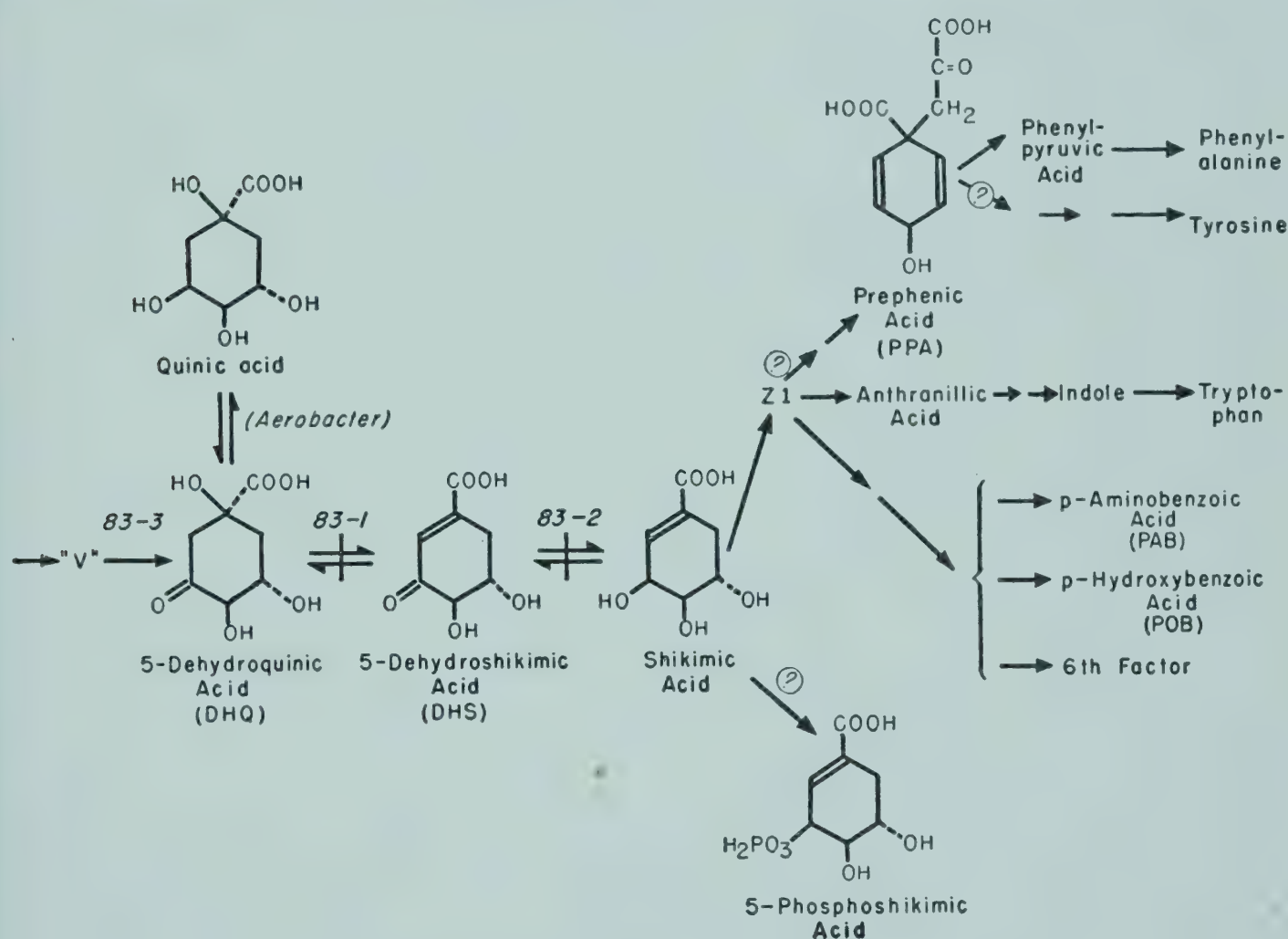


FIG. 1.

An opportunity for an experimental approach to this problem arose from the chance isolation of several aromatic polyauxotrophs of *Escherichia coli* (1) and one of *Neurospora* (17), i. e., mutants that required a mixture of four aromatic compounds. These were tyrosine, phenylalanine, tryptophan, and *p*-aminobenzoic acid (PAB).

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Subsequent work showed that most of the bacterial strains had in addition a relative requirement for traces of a fifth aromatic growth factor, *p*-hydroxybenzoic acid (POB) (2); and under certain circumstances they also required traces of a sixth factor, as yet unidentified.

#### ACCUMULATED COMPOUNDS; NUTRITIONAL STUDIES

*Shikimic acid.* The common aromatic structure of these several required compounds led to an empirical search for a possible common precursor that could replace them. After fruitlessly testing some 55 aromatic and hydroaromatic compounds, we had the good fortune to be given by Dr. R. Stanier a sample, prepared by Dr. H. O. L. Fischer, of a rare plant acid, shikimic acid. This compound was found to be active for certain of the bacterial mutants (3), and Tatum independently found it to be active for his *Neurospora* mutant (17). Furthermore, the growth response of the bacterial strains was proportional to shikimic acid concentration, and was approximately the same as that produced by a molar equivalent of the required mixed aromatic supplement (in correct proportions). Finally, other bacterial strains, blocked in a later reaction in the same sequence, could be shown to accumulate shikimic acid in their culture filtrates (3). In view of these findings, we concluded that this compound is a precursor of the aromatic cell constituents required by these mutants. Our identification of the accumulated shikimic acid was based on chromatographic and microbiological evidence; subsequently Shigeura and Sprinson isolated the compound (15). Furthermore, they showed that in a growing *E. coli* culture, forming tyrosine freely from glucose, added labeled shikimic acid was the source of 18 per cent of the tyrosine formed.

*5-Dehydroshikimic acid.* Using the technique of syntrophism (cross-feeding between different strains) on solid media, it was found that mutants blocked immediately before shikimic acid accumulated a precursor that was a growth factor for strains with still earlier blocks. This precursor, which was provisionally called Compound X, was accumulated in concentrations of several hundred



mg. per liter. Using the microbiological response to follow the course of purification, Dr. Ivan Salamon isolated the compound with the aid of charcoal chromatography. It turned out to be a reducing substance that was labile to heat or alkali and had a pronounced ultraviolet absorption peak ( $\epsilon = 11,900$ ) at 234 m $\mu$ . It was identified as 5-dehydroshikimic acid (DHS) (14).

*5-Dehydroquinic acid.* The next precursor back was much slower to be recognized, since it showed no growth-factor activity for mutants blocked before it. This problem was solved in two stages. First, studies on certain related problems revealed striking preferential synthesis in the aromatic series. To be more explicit, it was found that a slight partial block in this sequence first interfered with the synthesis of tyrosine, while the other aromatic metabolites continued to be made; then, with increasing completeness of the block, additional requirements appeared successively for phenylalanine, tryptophan, PAB, and POB (4). Detection of a weak growth factor would therefore be favored by the presence of tyrosine and phenylalanine, and so the mutants blocked before DHS were retested for syntrophism under these conditions. Certain of these strains were then found to accumulate a substance, Compound W, to which others did show a response. However, this response was barely detectable. In the second stage of development of this problem, this poor response was converted to a good one by means of a secondary mutation. This was accomplished by screening a large number of cells on Compound W, and selecting a rare secondary derivative that responded well to this substance, presumably through an improvement in accessibility of exogenous Compound W to its enzyme (7). This secondary mutant could conveniently be used for bioassay during purification of Compound W. Dr. Ulrich Weiss then isolated the compound by means of charcoal chromatography and precipitation as the brucine salt. It was found to be heat- and alkali-labile, like DHS, but to lack a pronounced UV absorption spectrum. It was identified as 5-dehydroquinic acid (DHQ) (18).

*Quinic acid.* A closely related compound, quinic acid, which is



known to be rather broadly distributed in the plant kingdom, has been reported by Gordon et al. (8) to be a growth factor in high concentration for a *Neurospora* mutant, and so has been suggested as a possible intermediate in aromatic biosynthesis. Furthermore, we found that the *Aerobacter* secondary mutants that responded well to DHQ responded equally well to quinic acid (7). However, *E. coli* mutants that responded to DHQ neither responded to quinic acid nor accumulated it. Furthermore, we were unsuccessful in a search for an *Aerobacter* auxotroph blocked between quinic acid and DHQ. For these reasons we provisionally considered that quinic acid is not an intermediate in the aromatic path, but rather is introduced into the path by an adventitious enzyme present in *Aerobacter* (7). This view has now been supported by the recent finding, to be elaborated on later in this paper, that this enzyme, quinic dehydrogenase, is present in extracts of wild-type *Aerobacter* but absent from those of several other organisms that can synthesize their aromatic amino acids (10). In contrast, these organisms (which include *E. coli*) all yielded the enzymes that interconverted DHQ, DHS, and shikimic acid (11, 20). If quinic acid were an equally obligatory intermediate, one would expect quinic dehydrogenase to have a similarly wide distribution.

*Isotopic studies.* This is as far back as we have been able to penetrate by the method of searching for growth factors that could replace the aromatic supplement. Accordingly, in order to throw light on earlier stages in the aromatic path, we have collaborated with Dr. David Sprinson in studies on shikimic acid formation from various isotopically labeled carbon sources. The results obtained could not be explained in terms of known paths of glucose metabolism, and therefore pointed to the existence of an important unknown path. This work will be discussed by Dr. Sprinson elsewhere in this symposium.

*5-Phosphoshikimic acid and Compound Z1.* At the other end of the chain, beyond shikimic acid, we have encountered three accumulated compounds that are completely devoid of nutritional activity for any mutant; they were recognized through the fact that they yielded active growth factors on autoclaving.



One of these compounds is found, in large or small quantities, in filtrates of all mutants that accumulate shikimic acid. It is stable to heating with alkali, but on acid hydrolysis it was found to yield shikimic acid (6). Weiss has isolated this accumulated compound as a potassium salt and identified it as 5-phosphoshikimic acid. The position of attachment of the phosphate group was established by exclusion: sensitivity to destruction by periodate showed that the material could not be 4-phosphoshikimic acid; and it was shown to differ in several respects from a sample of 3-phosphoshikimic acid. The 3-phosphoshikimic acid was synthesized from shikimic acid after protecting, by acetonide formation, the 4- and 5-hydroxyl groups, which are *cis* to each other and *trans* to the 3-hydroxyl. 5-Phosphoshikimic acid requires prolonged autoclaving with relatively strong HCl for maximal yield of shikimic acid, which is only about 50 per cent of that expected. Hydrolysis by potato phosphatase, in contrast, is complete, and has proved useful for assay purposes.\*

The second inactive compound, Z1, also is stable to heating with alkali and yields shikimic acid on heating with acid. It is much more labile to acid than is 5-phosphoshikimic acid. Some mutants accumulate large amounts of Z1 together with traces of shikimic acid and phosphoshikimic acid, while others accumulate the latter two compounds and no Z1 (6). It is therefore clear that Z1 arises at a later stage in this biosynthetic chain than the other two compounds. To distinguish these three compounds in filtrates, the latter were chromatographed on paper, which was then sprayed with dilute HCl and heated to release shikimic acid from the inactive compounds. The spots of shikimic acid were then detected by bioautography with an appropriate mutant. Phosphoshikimic acid, as would be expected from its polarity, moved much less rapidly than shikimic acid in butanol-formic acid. Compound Z1, in contrast, moved more rapidly than shikimic acid; one can therefore infer that it possesses, attached to shikimic acid, some organic residue which decreases its polarity.

\* We are grateful to Dr. A. Kornberg for a sample of potato phosphatase and for directions for its preparation.



*Prephenic acid.* The third inactive compound, prephenic acid (PPA), is accumulated by *E. coli* mutants that require phenylalanine or phenylpyruvic acid for growth. PPA has the unusual property of giving rise, in the presence of acid, to an active growth factor for the same phenylalanine auxotroph that accumulated it (5). The degree of this lability is extraordinary: the half-life of PPA at room temperature is only 130 hours at pH 7, 13 hours at pH 6, and a few minutes at pH 1-2. This property explains a very puzzling observation made several years ago by Simmonds (16): namely, that a phenylalanine auxotroph, after sufficiently prolonged cultivation, accumulated large quantities of a growth factor for itself. We have found that such mutants first accumulate PPA, and then later, unless precautions are taken to keep the culture alkaline, the concentration of PPA decreases while that of active growth factor rises. The accumulation of PPA and its lability have been independently discovered by Katagiri and Sato in Japan (9, 9a).

PPA was partly purified by charcoal chromatography in alkaline solution. An active eluate, placed in a spectrophotometer, showed only end absorption in the ultraviolet. On addition of HCl a sharp peak at 290 m $\mu$  developed in the course of 2 hours, and on addition of alkali after HCl the peak shifted instantly to 320 m $\mu$ . Authentic phenylpyruvic acid was found similarly to produce a peak slowly at 290 in acid and instantaneously at 320 in alkali; but the difference was that phenylpyruvic acid reacted directly with alkali to form the enolate that absorbed at 320 m $\mu$ , while PPA required treatment with acid before it would undergo this reaction. It appeared that the acid treatment first converted PPA to phenylpyruvic acid, and then slowly converted this compound to an unknown tautomer absorbing at 290 m $\mu$ . This conclusion was confirmed by isolating the dinitrophenylhydrazone of the phenylpyruvic acid formed from PPA (5).

PPA was isolated as a barium salt and partly identified by Dr. Weiss. It was found to be a nonaromatic cyclic compound (19). The identification was completed by Dr. Gilvarg, who will present this work in a separate contribution to this symposium.



## ENZYMATIC STUDIES

I should now like to describe some studies on certain enzymes in this biosynthetic sequence. These enzymes have been obtained from bacteria in cell-free solution by grinding with glass powder or by sonic oscillation.

First of all, PPA can be converted to phenylpyruvic acid not only by acid, but also by extracts of the wild type. In contrast, this reaction is not catalyzed by extracts of some phenylalanine auxotrophs. These enzymatic observations are particularly important because of the complete nutritional inactivity of PPA; their significance will be discussed below.

*5-Dehydroshikimic reductase.* The reduction of DHS to shikimic acid has been studied by Yaniv and Gilvarg (20). The enzyme for this reaction, 5-dehydroshikimic reductase, is specific for TPN as hydrogen carrier. The reaction, which is reversible, has been followed by coupling the oxidation of shikimic acid with the reduction of oxidized glutathione, and measuring the resulting SH production. The enzyme has been purified about 10-fold by treatment with  $\text{MnCl}_2$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and calcium phosphate gel. It exhibits no cofactor or ionic requirement other than TPN. The pH optimum is 8.5, and the Michaelis constants for TPN and shikimic acid, determined at this pH, were  $3.1 \times 10^{-5}$  and  $5.5 \times 10^{-5}$  M., respectively. The equilibrium constant was such that the O/R potential of DHS/shikimic acid would equal that of  $\text{TPN}^+/\text{TPNH}$  at about pH 8.5.

The wild type yielded approximately the amount of DHS reductase required to account for its growth rate, and a mutant blocked between DHS and shikimic acid yielded none.

*5-Dehydroquinase.* 5-Dehydroquinase, which removes a molecule of water from DHQ to form DHS, has been similarly investigated by Mitsuhashi (11). Its activity is readily measured by virtue of the fact that DHS has a high absorption peak at 234  $\text{m}\mu$ , whereas DHQ does not absorb light at this wave length. Activity is strictly proportional to enzyme concentration. This enzyme has also been purified



about 10-fold. No cofactor requirement could be demonstrated; the enzyme was equally active in tris-(hydroxyethyl)-aminomethane-HCl buffer or in potassium phosphate buffer.

The reaction is reversible, with the equilibrium constant ( $\text{DHS/DHQ} = 15$ ) heavily in favor of DHS, presumably because dehydration is favored by conjugation of the resulting double bond with a carbonyl group. The Michaelis constant for DHQ is low ( $4.4 \times 10^{-5} \text{ M.}$ ), which may be interpreted as evidence that DHQ is the physiological substrate of the enzyme. The pH optimum is 8.0.

The wild type yielded approximately the amount of dehydroquinase required to account for its growth rate. In contrast, no enzyme could be detected in extracts of mutants completely blocked in this reaction; the test would have detected a concentration 1/300 that of the wild type. The presence of an inhibitor was excluded by showing that mixtures of wild-type and mutant extracts were precisely as active as the wild-type extract alone (12). Finally, mutants that were incompletely blocked in this reaction, as shown by their growth requirements, yielded intermediate quantities of the enzyme (12).

*Quinic dehydrogenase.* Though we have presented evidence that quinic acid is not directly on the aromatic path, it is so closely related to this path that we might consider briefly the properties of the enzyme that converts it to DHQ. This enzyme, quinic dehydrogenase, was studied by Mitsuhashi (10). As was noted above, it is present in wild-type *Aerobacter*, but not in *E. coli*.

Quinic dehydrogenase activity was followed by measuring at 340  $\text{m}\mu$  the reduced pyridine nucleotide formed in the reaction. This enzyme, in contrast to DHS reductase, is specific for DPN. The reaction is reversible. The enzyme shows no cofactor or ionic requirement other than DPN. The pH optimum is high, about 9.8, and the Michaelis constants for quinic acid and DPN are  $4.9 \times 10^{-4}$  and  $1.4 \times 10^{-5}$ , respectively. The equilibrium constant was such that the O/R potential of DHQ/quinic equalled that of  $\text{DPN}^+/\text{DPNH}$  at about pH 9.7.

Crude extracts reacted nearly as rapidly with TPN as with DPN.

but the activity of TPN was shown to be due to the presence in these extracts of nucleotide transhydrogenase plus catalytic amounts of DPN. As a result of this connection between DPN and TPN, a series of four reactions links quinic acid to shikimic acid. The equilibrium constants for these reactions yield a calculated overall shikimic-quinic equilibrium constant (reaction 5) of 1.0. This value was confirmed experimentally, allowing the series to reach equilibrium after starting with either quinic or shikimic acid (10).

Reaction		Enzyme	Equilibrium constant *
(1)	Quinic acid + DPN <sup>+</sup> ⇌ DHQ + DPNH + H <sup>+</sup>	Quinic dehydrogenase	3.0 × 10 <sup>-10</sup>
(2)	DHQ ⇌ DHS + H <sub>2</sub> O	Dehydroquinase	15
(3)	DPNH + TPN <sup>+</sup> ⇌ DPN <sup>+</sup> + TPNH	Nucleotide transhydrogenase	0.7
(4)	DHS + TPNH + H <sup>+</sup> ⇌ Shikimic acid + TPN <sup>+</sup>	DHS reductase	3.2 × 10 <sup>8</sup>
(5)	Quinic acid ⇌ Shikimic acid + H <sub>2</sub> O	Overall	1.0

\* The equilibrium constants for reaction (2) and (5) neglect the H<sub>2</sub>O term.

Because the coupled reactions from quinic acid to shikimic acid reoxidize DPNH, the technique for measuring quinic dehydrogenase described above, based on DPNH formation, is applicable only to preparations whose oxidation of quinic acid is much more rapid than their coupled reduction of DHS to shikimic acid. However, with other preparations, quinic dehydrogenase activity can be measured by a procedure that is more general but less precise; this procedure is based on a bioassay that measures DHS or shikimic acid but not quinic acid.

*Early reactions; Compound V.* Finally, I should like to mention some enzymatic studies that have opened up the possibility of extending the analysis of aromatic biosynthesis further back, into the no-man's-land where isotopic studies have failed to provide a detailed answer. As was noted above, we could detect no syntrophism among the various mutants blocked before DHQ, either because they were all blocked in the same reaction, or because any compounds accumu-



lated in their filtrates might, like Z1 or PPA, lack growth-factor activity. Nevertheless, it seemed likely that the organisms would accumulate precursors, since all the mutants with later blocks in this series accumulated precursors in generous quantities. Furthermore, since crude bacterial extracts had been found to convert known precursors into later members of the sequence, it seemed possible that such extracts might similarly convert even earlier, unknown precursors into later, detectable members of the sequence. Dr. Edwin Kalan undertook a test of this hypothesis.

For this purpose cell-free extracts of mutant 83-2, blocked immediately after DHS, were used, rather than those of mutants blocked after an earlier precursor, DHQ. This choice was based on the expectation that the favorable 15/1 equilibrium constant for DHS/DHQ would drive forward any series of reactions leading to DHQ formation. It was indeed found that filtrates of a mutant blocked before DHQ (strain 83-3), when incubated with an extract of strain 83-2, yielded respectable quantities of DHS, amounting to about 20 mg./l. This substance was determined by bioassay; it was identified by paper chromatography and by growth-factor activity (and inactivity) for appropriate mutants. The reaction is specific with respect to both enzyme and filtrate: extracts of a mutant blocked before DHQ failed to form DHS from an active filtrate (of the same organism), and an active extract of mutant 83-2 failed to form DHS from filtrates of the wild type. It therefore appears that mutants blocked before DHQ accumulate one or more compounds that can serve as precursors of the known members of this chain. We have provisionally designated the accumulated compound closest to DHQ as Compound V.

The success of this venture encouraged us to try something that we had been discussing for a long time with Dr. Sprinson: namely, the possibility that one might get a cell-free system to carry out the chain of reactions from carbohydrates to shikimic acid or a related compound. Accordingly, a number of carbohydrates were tested with the extract of strain 83-2 just described. The experiments were successful. They have opened up the entire early part of the aromatic



sequence to enzymatic analysis; furthermore, they make it possible to test phosphorylated and other intermediates that might not penetrate into the intact cell. This work was carried out by Dr. Kalan, and will be presented by him.

### CONCLUSIONS

And now, let us consider the metabolic role of the compounds described.

I am confident that DHQ and DHS are true, obligatory intermediates in aromatic biosynthesis, since (a) they can be used by the cells to synthesize aromatic metabolites; (b) the conversion of one to the other is effected by a single enzyme; and (c) absence of this enzyme is associated with a requirement for these aromatic metabolites. The conclusion that the enzyme is single is not inferred from the purification achieved, which is quite modest (around 10-fold); this conclusion is rather based on the nature of the reaction, and on the fact that no cofactor requirement could be demonstrated. For on structural grounds the only plausible alternative to a one-enzyme reaction would seem to be a series of reactions involving interaction with a cofactor.

An identical series of considerations, based on the studies of the enzyme converting DHS to shikimic acid, leads us to conclude that shikimic acid is also an obligatory intermediate in this sequence.

Quinic acid, for reasons that have been given above, is not considered to be directly on the path. Phosphoshikimic acid is provisionally put down in the scheme also as a side-product, simply because in our experience with 59 mutants blocked somewhere along the chain of common precursors, not one has been found to be blocked between shikimic acid and phosphoshikimic acid. The limited data available on Compound Z1 are compatible with assigning it a position on the path, but this decision must also be considered provisional until the formation and utilization of this compound are investigated enzymatically.

Despite the complete nutritional inactivity of PPA, we have assigned this compound a definite position before phenylalanine



because of the evidence cited above for its enzymatic conversion to phenylpyruvic acid by extracts of the wild type and not by those of a phenylalanine auxotroph. These observations also establish phenylpyruvic acid as a true, obligatory intermediate. This evidence is important, since the transaminase that converts phenylpyruvic acid to phenylalanine, which has been obtained from *E. coli* by Rudman and Meister, was found to act also on *p*-hydroxyphenylpyruvic acid and several other  $\alpha$ -keto acids (13). Furthermore, no mutant deficient in this enzyme has been reported. In consequence, if we did not have information on the reaction preceding phenylpyruvic acid the growth-factor activity of this compound could have been attributed to non-specific transamination.

On structural grounds, PPA is a reasonable precursor also of tyrosine, as will be learned from Gilvarg's presentation; furthermore, it is accumulated by certain tyrosine auxotrophs as well as by phenylalanine auxotrophs. However, we cannot definitely place PPA on the path to tyrosine until enzymatic support for this view has been obtained.

I shall not discuss the path to tryptophan, which is being covered elsewhere in this symposium. It might be noted that several workers have been led, by observations involving the use of growth inhibitors, to infer that tryptophan, tyrosine, and phenylalanine can be interconverted in *E. coli*. The results described in this paper are clearly in conflict with such a view. Indeed, there are increasing indications that reversal of growth inhibition is sometimes based on interference with penetration of the inhibitor into the cell, rather than on the metabolic role that is conventionally inferred for reversing agents in the methodology of inhibition analysis. It therefore seems that this methodology can furnish only suggestive evidence concerning metabolic paths.

As was noted above, dehydroquinase and DHS reductase have been found in a variety of microorganisms and plants that can synthesize their own aromatic amino acids; furthermore, they are absent from an animal tissue, which requires these aromatic compounds. In addition, though shikimic acid has been isolated in the past from

very few plants, we have been able, by the sensitive method of bioautography, to detect traces of this compound in all of a number of plant extracts examined. It is therefore clear that the path described here is widespread in its distribution; and it may well be the only path present in the biological kingdom for synthesizing the benzenoid amino acids, and a number of other aromatic metabolites, from non-aromatic sources.

The work that I have summarized here has resulted from the joint effort of a number of investigators. In addition to the references to colleagues that have already been made, I should like to acknowledge particularly the excellent technical assistance of Mrs. Mingioli, the very pleasant and fruitful collaboration with Dr. Sprinson and his colleagues, Shigeura, Sprecher, and Srinivasan; and finally the recent critical and catalytic contributions of Dr. Gilvarg to the work of the rest of our group.

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# PREPHENIC ACID AND THE AROMATIZATION STEP IN THE SYNTHESIS OF PHENYLALANINE \*

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IT WAS SIMMONDS who first observed that a phenylalanine-requiring mutant of *Escherichia coli* accumulated a growth factor for itself (4). Later Davis (1), and independently Katagiri and Sato (3) identified this growth factor as phenylalanine and showed that it had arisen from an unstable compound that had accumulated in the growth medium. This compound, prephenic acid (PPA), is exceedingly acid-labile; unless the pH of the culture is maintained above 7, PPA is spontaneously transformed to yield phenylpyruvic acid (1). This product is in turn converted by the mutant to phenylalanine, which is partly used for further growth and partly excreted into the medium.

Since phenylpyruvic acid has an absorption maximum at 320 m $\mu$  in strong alkali, it was possible to assay for PPA by first converting it to phenylpyruvic acid by warming with *N*/10 HCl. The solution was then brought to pH 14, and the PPA concentration was determined from the absorption at 320 m $\mu$  of this solution compared with the PPA solution alkalized directly.

In order to isolate prephenic acid, culture filtrates of mutant 83-5 were brought to pH 9.0 and passed through a column of charcoal (Nuchar c-190 45 g. per liter of medium). The PPA was eluted

\* A preliminary communication concerning this work has appeared (7).

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with water, and the barium salt of PPA was precipitated from methanol-water. Repeated precipitation from methanol-water yielded a crystalline salt.<sup>1</sup> The elementary analysis agreed with the empirical formula  $C_{10}H_8O_6Ba \cdot H_2O$ .

Found	C 31.66	H 2.82	Ba 36.02
Calculated	31.64	2.66	36.19

The acid-catalyzed conversion of PPA to phenylpyruvic acid was found to be accompanied by the release of one equivalent of  $CO_2$ . This observation and the empirical formula indicate that PPA is a  $C_{10}$  dicarboxylic acid.

This finding is in harmony with earlier observations on the origin of the side chains of phenylalanine and shikimic acid. This earlier work had shown that the  $\beta$ -carbon of the side chain of phenylalanine and tyrosine is derived, to the extent of about 50 per cent, from carbon 1 of glucose (2), whereas the carboxyl carbon of shikimic acid is derived from the 3,4 carbons of glucose (5). Thus in the conversion of shikimic acid to phenylalanine the carboxyl carbon of shikimic acid is lost. It seemed clear that in this transformation PPA, a  $C_{10}$ -dicarboxylic acid, is an intermediate in which both the side chain and the carboxyl group coexist attached to the ring; and it is in the conversion of PPA to phenylpyruvic acid that the loss of the former carboxyl carbon of shikimic acid occurs.

Isotopic evidence makes it possible also to locate the point of attachment of the two side-chains on the ring of PPA. In shikimic acid obtained from organisms grown on labeled glucose, C-1 of glucose is found principally in position 2 of the ring and C-6 of glucose in position 6 (5). Similarly, in the ring of phenylalanine and that of tyrosine, respectively, C-1 of glucose (2) and C-6 of glucose (6) are found principally in the ortho position. It therefore appears that the carboxyl group of shikimic acid and the side chain of phenylalanine (or tyrosine) are both attached to carbon 1 of the

<sup>1</sup> The purification of PPA was largely the work of Dr. U. Weiss and Mrs. E. Mingioli.



ring in PPA.<sup>2</sup> The resulting quaternary structure of this carbon atom would exclude an aromatic structure for PPA.

Additional proof for non-aromaticity is furnished by two further facts: (a) PPA takes up 3 to 4 molar equivalents of  $H_2$  over Pt at room temperature; and (b) after reduction of the carbonyl group of the side chain of PPA, the resulting compound takes up two molar equivalents of  $Br_2$ .

The product of hydrogenation spontaneously forms a lactone on acidification, as shown by a positive alkaline hydroxamic test. This reaction provides additional evidence that the carboxyl carbon is located on carbon 1 of the ring, for in this position it would form a  $\gamma$ -lactone with the  $\alpha$ -hydroxyl group of the reduced side chain.

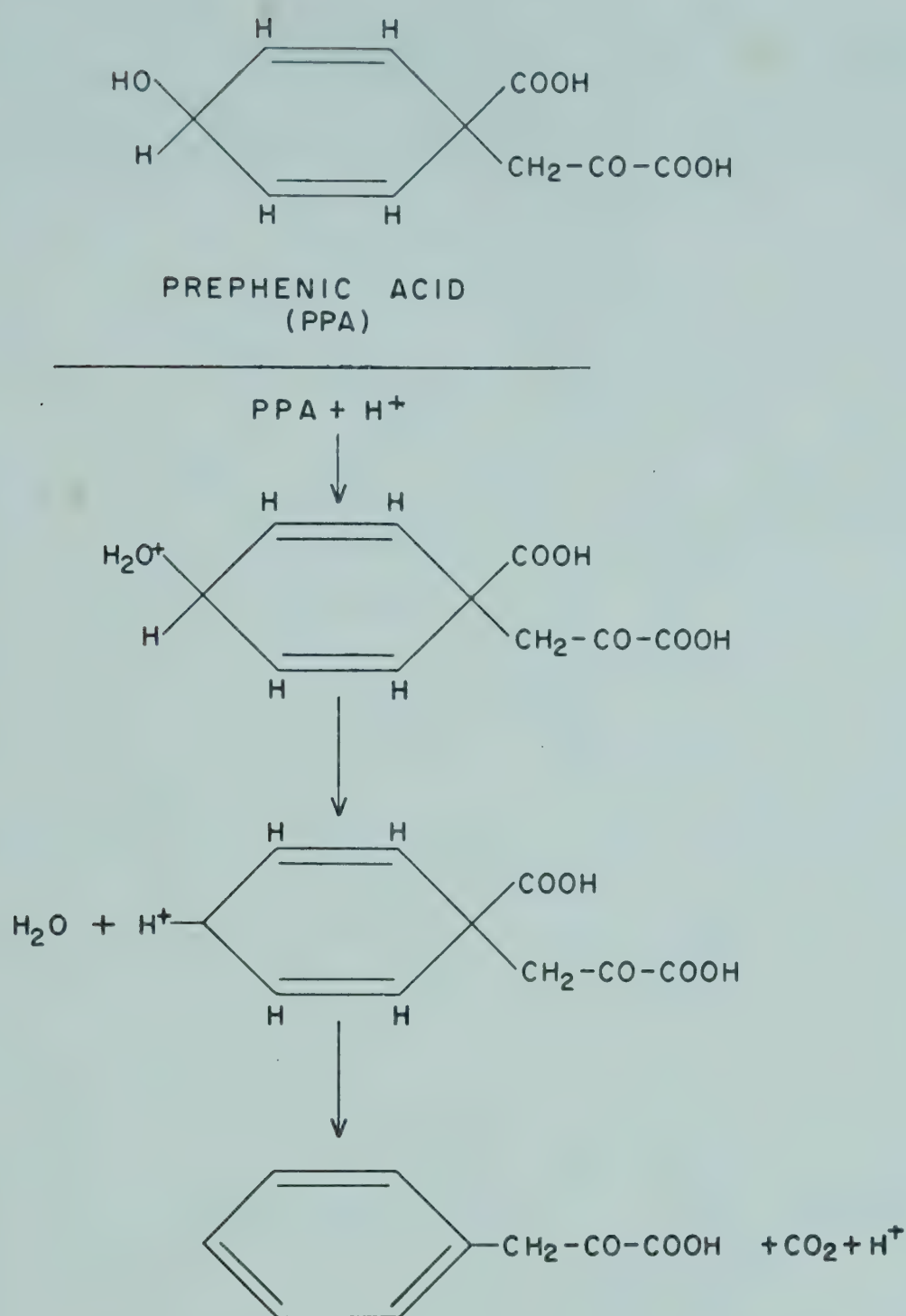
The reduction of the carbonyl group was effected by  $NaBH_4$ . The product, prephenyllactic acid, like PPA, decarboxylates upon acidification; this reaction yields phenyllactic acid. Prephenyllactic acid has a molar extinction coefficient at  $260\text{ m}\mu$  so low ( $\epsilon = 20$ ) as to be incompatible with either aromaticity or the presence of a conjugated double bond system in the ring.

Since all the valences of carbon 1 of the ring have been accounted for, there cannot be a double bond in the 1-2 or 1-6 position. This requirement, and the spectral considerations which exclude a conjugated double-bond system, place the double bonds in the 2-3 and 5-6 positions in the ring.

In order to account for the elementary analysis and to explain the acid catalysis of aromatization of PPA, it is necessary to postulate the presence of an hydroxyl group on the ring. These considerations lead to the following proposed structure for PPA.

The acid-catalyzed conversion is visualized as proceeding in the following manner:

<sup>2</sup> This argument assumes that the biosynthetic path to tyrosine and phenylalanine is identical in *Saccharomyces cerevisiae* and in *E. coli*; that the side-chain of each of these two amino acids is attached to the same position on the ring; and that there is no migration of the side-chain in proceeding from shikimic acid to PPA, or from PPA to phenylalanine.



More direct evidence for the existence of a para-hydroxyl group was obtained when it was found that heating in 2 *N* NaOH at 100° C. for 5 minutes quantitatively converted PPA to *p*-hydroxyphenyllactic acid. The alkali labilizes the double bond of the enolate form of the side-chain; and the resulting aromatization yields a product different from that formed by the acid-catalyzed aromatization.

In a consideration of the origin of the carbon skeleton of PPA it appears that the ring and the carboxyl carbon are derived from





# THE BIOSYNTHESIS OF SHIKIMIC ACID FROM LABELED CARBOHYDRATES \*

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THE ELEGANT STUDIES of B. D. Davis brought the problem of the biosynthesis of the aromatic amino acids to a new level of knowledge and interest with the discovery in 1950 that shikimic acid is an intermediate in their formation (1).<sup>1</sup> The diversity and importance in biological materials of aromatic compounds, from simple hydrocarbons to complex alkaloids, has stimulated several theories of their biogenesis on the basis of structural relationships or known laboratory reactions. Until recently, however, direct experimental attack on this problem was lacking. Some progress has recently been made by the studies on the formation of tyrosine and phenylalanine from labeled compounds (4-7). These amino acids, however, in contrast to shikimic acid, do not permit the two sides of the ring to be distinguished from each other. Accordingly, an investigation, in collaboration with Davis, of the biosynthesis of shikimic acid from labeled compounds appeared to be a challenging opportunity.

## THE UTILIZATION OF D-GLUCOSE

This task was first undertaken in our laboratory by H. T. Shigeura, who worked out methods for the isolation of shikimic acid from filtrates of *Escherichia coli* mutant 83-24, and for its chemical degradation (8). It was observed that when this organism was

\* This work was supported by grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council), the Lederle Laboratories Division of the American Cyanamid Company, the National Institutes of Health, United States Public Health Service, and the Rockefeller Foundation.

<sup>1</sup> It is of historical interest that 116 years ago quinone was first discovered by



grown on a glucose-salts medium with the addition of  $\text{NaHC}^{14}\text{O}_2$ ,  $\text{HC}^{14}\text{OONa}$ , or acetate labeled in either carbon atom, the activity incorporated into shikimate from these additions was negligible. The acetate had been metabolized, since the  $\text{CO}_2$  evolved during growth was quite active. The non-participation of intermediates of the tricarboxylic acid cycle in the biosynthesis of tyrosine phenylalanine in yeast had also been found by Gilvarg and Bloch (6), who showed that although labeled acetate, in the presence of glucose, was incorporated into glutamate, aspartate, and alanine, no activity was observed in the aromatic amino acids.

TABLE 1  
CONTRIBUTIONS OF INDIVIDUAL CARBON ATOMS OF  
GLUCOSE TO SHIKIMATE FORMATION

Label in glucose	COOH	Percentage of activity of glucose in shikimate carbon atoms					
		C-1	C-2	C-3	C-4	C-5	C-6
C-1	1	0	60	0	1	5	0
C-2	2	50	9	23	2	21	0
C-3 or -4	85	0	0	37	83	39	0
C-6	12	0	0	0	0	0	100
C-5 (calc.)	0	50	30	40		30	0

When labeled glucose was utilized for shikimate synthesis it gave the distribution of activities shown in Table 1 and Fig. 1 (9). The value for each shikimate carbon atom is represented as the percentage of the activity of the labeled atom of the glucose from which the shikimate had arisen. It may be observed<sup>2</sup> that S-4, 6, and 7 are essentially "accounted for" by G-3 (4) and 6, whereas S-1, 2, 3, and 5 show significant "deficiencies" that presumably arise from

Woskresensky (2) as a product of the oxidation by manganese dioxide and sulfuric acid of quinic acid, a compound closely related to shikimic acid. Wöhler (3) later found several simple aromatic compounds among the products of the dry distillation of quinic acid.

<sup>2</sup> The abbreviations S-1, S-2 . . . S-7 will be used to denote carbon atoms 1, 2 . . . and carboxyl of shikimate; and G-1, G-2 . . . to denote carbon atoms 1, 2 . . . of glucose. C-1, C-2 . . . refer to carbon atoms of other compounds.

G-5, the one carbon atom of glucose that it was not possible to test experimentally. Contributions from G-5 are calculated by difference and are, therefore, limited in accuracy by the cumulative errors of 4 separate degradations. S-6 is derived uniquely from G-6, while

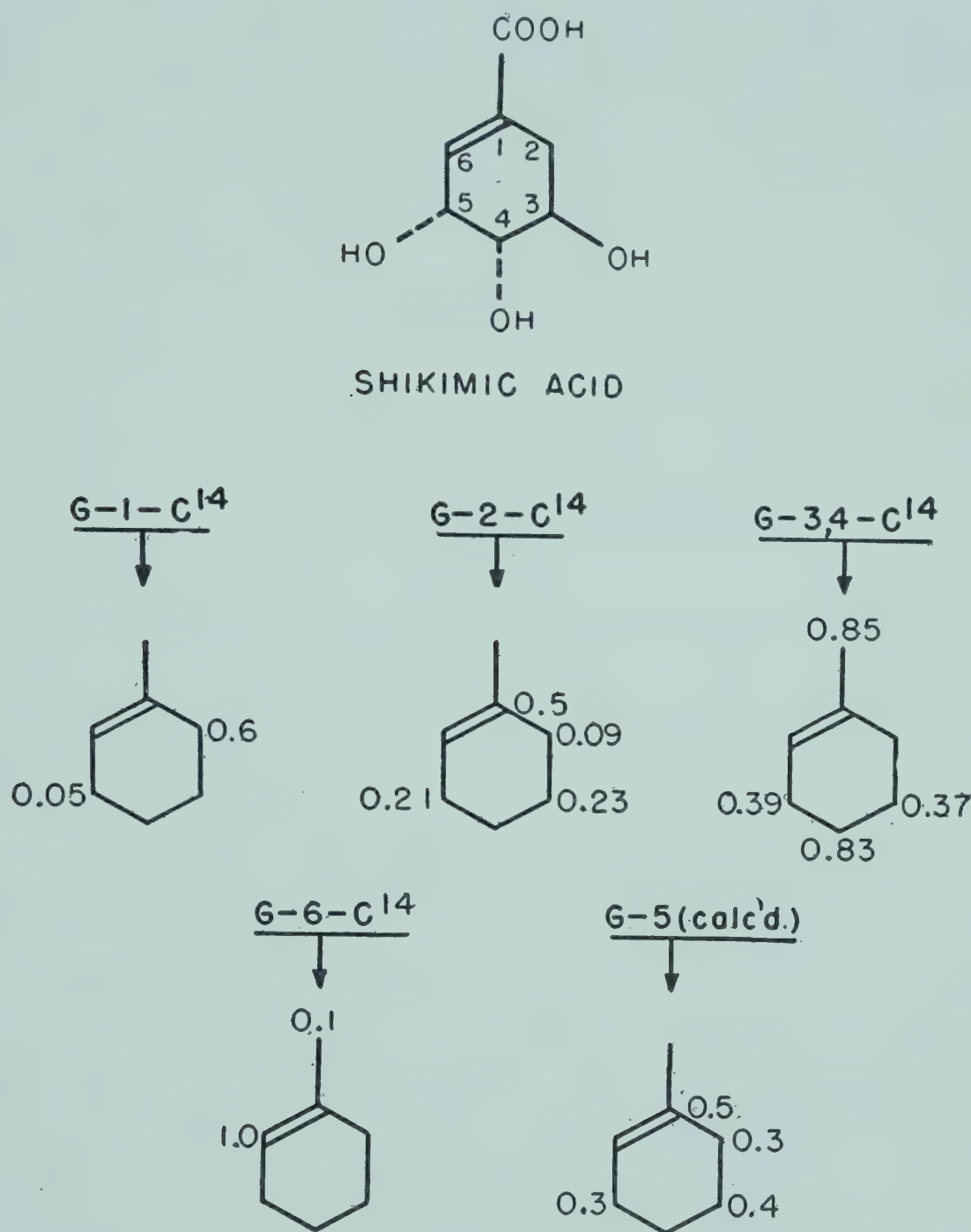


FIG. 1. Fractions of activity of carbon atoms of glucose incorporated into shikimic acid.

S-1 appears to be derived almost entirely from a mixture of G-2 and 5, and S-2 from G-1 and 5. S-3 and 5 are the most heterogeneous in origin, each being derived from at least 3 of the 4 middle carbon atoms of glucose, G-2, 3 (4), and 5. A summary of the most important incorporations is shown in Fig. 2.



A striking aspect of these findings is the complete lack of equilibration between G-1 and G-6. In the glycolytic pathway these carbon atoms are "equilibrated" (10) through the action of triose isomerase (as are also G-2 with 5 and G-3 with 4); and, as pointed out by Gilvarg (p. 00), this reaction of glycolysis is reflected in the synthesis of the side chain of tyrosine and phenylalanine, in which G-1 and G-6 each contribute about 50 per cent to the  $\beta$ -carbon atom.<sup>3</sup> The

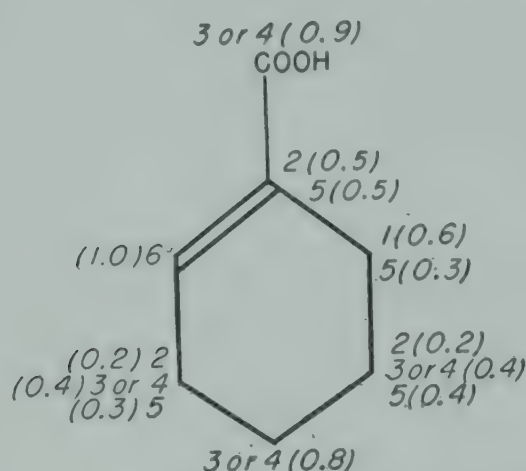


FIG. 2. Major contributions of glucose carbon atoms to shikimate biosynthesis.

separation of G-1 and G-6 in the ring of shikimate shows that the glycolytic pathway does not contribute toward its synthesis (unless a triose phosphate is used before isomerization). The relative positions of G-1 and G-6 in the ring also preclude cyclization of the intact carbon chain of glucose as a reaction in shikimate formation.

Since G-1 is extensively incorporated into S-2, the oxidative pathway of glucose metabolism (11-15) via pentose and sedoheptulose, which involves loss of G-1, cannot be the sole source of intermediates for shikimate synthesis. However, 7-carbon sugars might well be derived not only from pentose, but also by mechanisms that lead to incorporation of G-1. One possibility would be condensation of pentose with "active glycolyaldehyde," which has been shown to be derivable from C-1 and 2 of fructose-6-phosphate (16; cf. 14, 17).<sup>4</sup>

<sup>3</sup> It may be of interest to point out that the "alanine" side chain of tyrosine and phenylalanine, though evidently derived from a product of glycolysis, is not equilibrated with tricarboxylic acid cycle intermediates under conditions where alanine is (6).

<sup>4</sup> This 2-carbon fragment has also been shown to arise (13,14) from C-1 and C-2 of D-ribose-5-phosphate (via ribulose phosphate) and of sedoheptulose-7-phosphate, from D-erythrulose, and from hydroxypyruvate.

Another possibility would be transaldolization between sedoheptulose-7-phosphate and hexose diphosphate to give sedoheptulose-1,7-diphosphate (18).

The independent positions of G-1 and G-6 in sedoheptulose formation and in shikimate biosynthesis, and the reasonable expectation that a non-cyclic 7-carbon compound is utilized in the latter process, suggested that heptoses might be involved in the synthesis of shikimate. However, efforts to grow *E. coli* on sedoheptulose, and to stimulate shikimate accumulation by addition of this compound, were without success. This result is not surprising in view of the fact that heptose phosphate esters have so far not been observed to be formed by direct phosphorylation of free heptoses. For this reason we have recently shifted emphasis to the search for a system that would not be limited by the impermeability of whole cells to phosphate esters.

In another paper of this symposium, Kalan and Srinivasan report an extensive conversion of sedoheptulose-1,7-diphosphate to 5-dehydroshikimic acid by cell-free extracts. These results lend support to the suggestion that heptoses may be involved in shikimate biosynthesis. It should be pointed out, however, that the currently postulated reactions for forming heptoses from hexose (13, 14, 17, 18) cannot explain the complex distribution of activity in shikimate derived from labeled glucose (see Table 1). Since G-6 is the precursor of both C-7 of sedoheptulose and S-6, the cyclization of a heptose derivative to dehydroshikimate would imply a correspondence in activity also between C-1 of the straight chain precursor and S-7. However, C-1 of sedoheptulose is derived from G-1 or G-2 (13-18), while S-7 is derived almost entirely from G-3, 4. It must therefore be assumed either that heptoses can be made in the growing organism by still unrecognized reactions, or that unknown pathways in carbohydrate metabolism are involved in the biosynthesis of shikimate.



## THE UTILIZATION OF D-XYLOSE

In order to gain further insight into the role of sugars in shikimate biosynthesis, P. R. Srinivasan investigated D-xylose-1- $C^{14}$  as a sole carbon source in a shikimate accumulation.<sup>5</sup> The results, summarized in Fig. 3, show that C-1 of pentose is incorporated into S-2 to the extent of 0.6. (This incorporation is the same as that shown above for G-1). Appreciable activity is also found in S-3, 4, and 7.

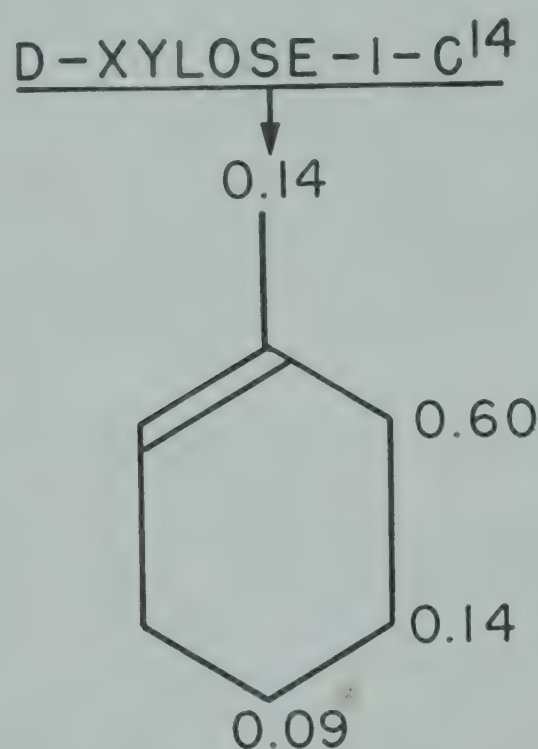


FIG. 3. Fractions of C-1 of D-xylose-1- $C^{14}$  utilized for shikimate formation.

With glucose as a precursor, these positions are derived partly or predominantly from G-3,4. In the conversion of pentose phosphate-1- $C^{14}$  (via sedoheptulose phosphate) to hexosemonophosphate by a liver (14) or a plant (20) enzyme preparation, the label is found almost entirely in G-1 and G-3, and in the ratio of 3:1, respectively. In accordance with these observations, the shikimate in Fig. 3 may be assumed to have been formed from pentose-1- $C^{14}$  by way of glucose-1,3- $C^{14}$ . It would appear that the activity of G-1 of this

<sup>5</sup> When glucose and pentose are both present in the medium, *E. coli* exhausts the glucose before utilizing the pentose (19). For this reason pentose was tested only in the absence of glucose. D-xylose was employed rather than D-ribose because this strain of *E. coli* could grow on the former but not the latter compound.

glucose was equal to C-1 of the pentose since, as pointed out previously, the utilization for S-2 was the same as in the glucose-1-C<sup>14</sup> accumulation (see Fig. 1). If this be the case, then by analogy with the results obtained with the enzyme preparations (14, 20), G-3 of the hypothetical glucose-1,3-C<sup>14</sup> was about 0.33 as active as C-1 of the pentose. Comparison of the incorporations from xylose (Fig. 3) with those from glucose-3,4-C<sup>14</sup> (Fig. 1) leads to the tentative conclusion that the contribution of G-3,4 to S-3 is essentially from G-3 ( $0.14/0.33 = 0.42$ , and  $0.42/0.37 \sim 1$ ), while that of G-3,4 to S-5 is only from G-4. On the basis of similar considerations the contribution from G-3,4 to S-7 is 50 per cent G-3 ( $0.14/0.33 = 0.42$ , and  $0.42/0.85 \sim 0.5$ ) and 50 per cent G-4, while the contribution of G-3,4 to S-4 is  $\frac{1}{3}$  G-3 ( $0.09/0.33 = 0.27$ , and  $0.27/0.83 = 0.33$ ) and  $\frac{2}{3}$  G-4.

Comparison of the xylose (Fig. 3) with the glucose-2-C<sup>14</sup> (Fig. 1) accumulation indicates that the dominant conversion of G-2 is distinct from that of C-1 of D-xylose, and suggests that while conversion to hexose is required for the utilization of pentose in shikimate formation, the conversion of hexose to pentose by loss of G-1 is a relatively minor pathway in the utilization of hexose.

#### ACETATE AND PYRUVATE AS SOLE CARBON SOURCES

Several careful studies are available on the biosynthesis of aromatic amino acids from labeled acetate (4) or pyruvate (7) as the sole carbon source. In general these observations may be interpreted as reflecting the reaction sequence: acetate  $\rightarrow$  pyruvate  $\rightarrow$  glucose  $\rightarrow$  shikimate  $\rightarrow$  tyrosine.

It has recently been shown that acetate can participate in a C<sub>2</sub> + C<sub>1</sub> condensation in *E. coli* (21), and furthermore, that the tricarboxylic acid cycle is substantially the only means available to this organism for combusting acetate (22). These investigations indicate that acetate-1-C<sup>14</sup> (as a sole carbon source) would first yield highly labeled pyruvate-2-C<sup>14</sup>, but after carboxylation of the pyruvate to oxaloacetate and only 5 turns in the tricarboxylic acid cycle the activity in carbon-2 would be diluted to 3 per cent of its original



level. Meanwhile, the  $\text{CO}_2$  produced by the cycle would be derived from both carbons of acetate, and fixation of this heavily labeled  $\text{CO}_2$  would yield pyruvate with high activity in the carboxyl group. Glucose, produced by a reversal of glycolysis, would be active in G-3,4, but G-1,2,5,6, would be derived almost entirely from the methyl carbon of acetate. The distribution of label from  $\text{C}^{13}\text{H}_3\cdot\text{C}^{14}\text{OOH}$  into tyrosine (4), interpreted in these terms, is in accordance with the observed conversion of glucose to shikimate (see Fig. 1), followed by the replacement on S-1 of the carboxyl of shikimate by the side-chain during tyrosine formation. This replacement is discussed in greater detail by Gilvarg elsewhere in this symposium). C-1, 2, and 6 of tyrosine would then be derived mostly from the methyl carbon of acetate; G-3,5 from a mixture of methyl and carboxyl; and C-4 from carboxyl (4).

It is noteworthy that several classes of alicyclic compounds, e. g., the carotenoids and steroids, in the biosynthesis of which an isoprene unit appears to be involved, are derived from acetate. On the other hand, the biosynthesis of the aromatic amino acids, and presumably the alkaloids and flavonoids (24) derived from them, is dependent on some reactions in carbohydrate metabolism which are as yet unknown and possibly unique for this purpose.

We are indebted to Dr. H. S. Isbell, National Bureau of Standards, for the glucose-1- $\text{C}^{14}$ , glucose-2- $\text{C}^{14}$ , and xylose-1- $\text{C}^{14}$ . The glucose-6- $\text{C}^{14}$  used in this investigation was a generous gift from Dr. G. Ehrensvärd.

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Further studies have disclosed that certain of the results reported above are unfortunately in error. The recent results indicate that S-2 is derived nearly equally from G-1 and G-6, suggesting that S-7,1,2 arises from the glycolytic pathway. It was also found that S-6 is derived only 0.5 from G-6, and partly from G-1.



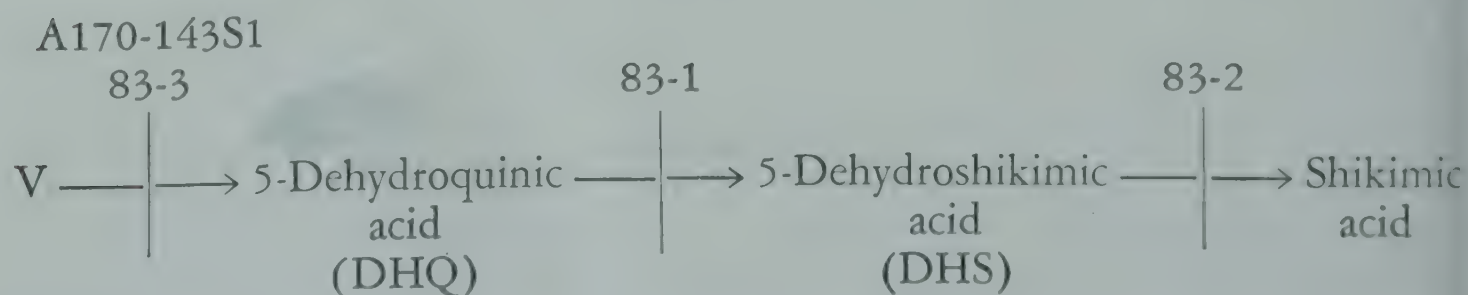
# SYNTHESIS OF 5-DEHYDROSHIKIMIC ACID FROM CARBOHYDRATES IN A CELL-FREE EXTRACT

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SPRINSON HAS just presented isotopic evidence that in the conversion of glucose to shikimic acid (and hence to the benzene ring of the aromatic amino acids) an unknown path of glucose metabolism must be involved. To study this path further we have used an enzymatic approach, and have attempted to convert carbohydrates, by means of a cell-free extract, to a known intermediate of aromatic biosynthesis.

The enzyme system used was an unfractionated cell-free extract of mutant 83-2 of *Escherichia coli*, which is blocked immediately after 5-dehydroshikimic acid (DHS) (see below). This organism was chosen, rather than one with an earlier block (e. g., 83-1), because the high DHS/DHQ equilibrium constant might be expected to drive forward any series of earlier reactions leading to DHQ formation. The DHS formed was estimated microbiologically with mutant A170-143S1, and it was identified as described by Dr. Davis elsewhere in this symposium in connection with the detection of Compound V.




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When a cell-free extract of strain 83-2, together with phosphate buffer at pH 7.4 and  $\text{MgCl}_2$ , was incubated with glucose, no significant formation of DHS could be detected. Incubation with various phosphorylated sugars, however, did result in the formation of DHS. The yield was found to have reached practically a maximum after 4 hours of incubation at 37° C.

TABLE 1

SYNTHESIS OF 5-DEHYDROSHIKIMIC ACID (DHS) FROM CARBOHYDRATES

Substrate	$\mu\text{M.}/\text{ml.}$	% Conversion (Molar)*	
		2 hrs.	4 hrs.
Glucose-6-Phosphate	5	3.8	5.4
Glucose-1-Phosphate	5	4.0	—
Fructose-6-Phosphate	5	4.6	—
Hexosediphosphate	5	4.0	5.6
Ribose-5-Phosphate	5	2.4	4.0
Glucose	5	0.4	—
6-Phosphogluconic Acid	5	0	0

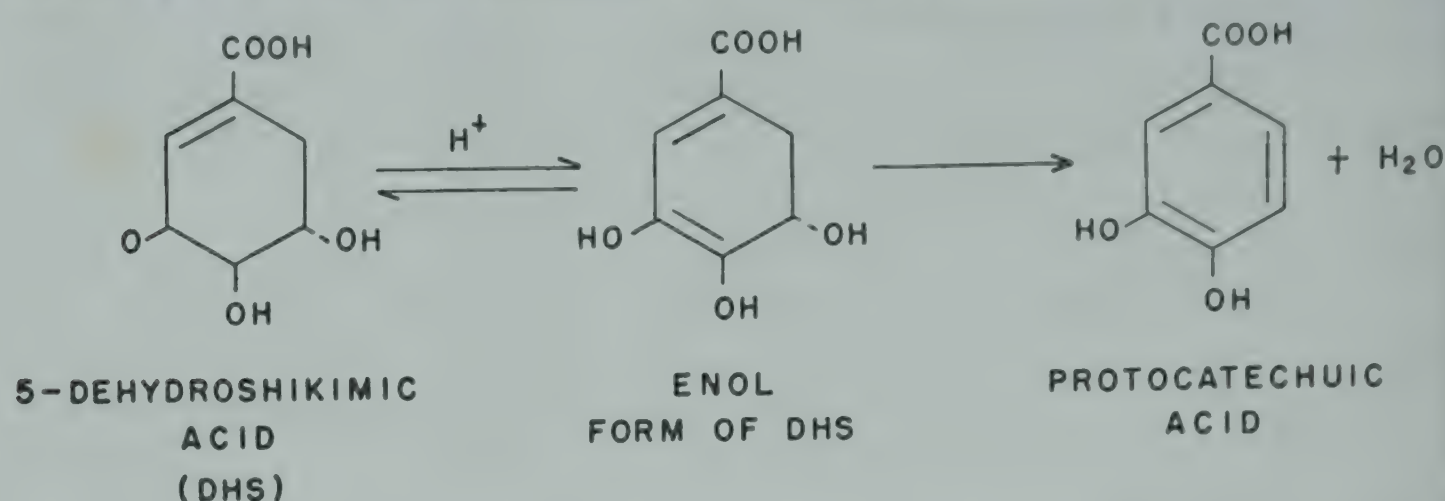
\* Average values of several experiments. System contained per ml. 5  $\mu\text{M.}$   $\text{MgCl}_2$ , 50  $\mu\text{M.}$  potassium phosphate buffer (pH 7.4), and 0.1 ml. of 83-2 extract containing 20 mg. of protein per ml.

Table 1 summarizes the results obtained with a variety of substrates. The phosphorylated hexoses, and also ribose-5-phosphate, yielded about 5 per cent of their molar equivalent of DHS. 6-Phosphogluconic acid was inactive, presumably owing to the fact, previously observed (2), that *E. coli* extracts contain very little TPN. Similarly, the previously noted inactivity of glucose is presumably due to a deficiency of hexokinase in these extracts.

Because of the small yield obtained, it was not certain that the carbon in these phosphorylated carbohydrates was actually being incorporated into DHS. This incorporation was therefore established by use of uniformly labeled  $\text{C}^{14}$ -glucose, which was incubated with ATP and hexokinase in order to convert to glucose-6-phosphate. An extract of mutant strain 83-2 was then added, and after 4 hours the reaction was stopped by adjusting the mixture to pH 1 with



6 *N* HCl. The DHS formed was measured and was then converted to protocatechuic acid according to the method of Salamon and Davis (4), after the addition of diluent protocatechuic acid. The



molar specific activities of the glucose and the isolated protocatechuic acid (Table 2) showed that at least 60 per cent of the carbon of DHS had been derived from the glucose added.

TABLE 2  
CONVERSION OF UNIFORMLY LABELED GLUCOSE TO DHS

Compound	Molar Activity	Per cent Conversion
Glucose	$2.5 \times 10^6$	
Protocatechuic acid	$1.5 \times 10^6$	60

The formation of DHS from glucose presumably requires at some stage a 7-carbon open-chain intermediate. One possibility for such an intermediate seemed to be sedoheptulose-7-phosphate, in view of the widespread distribution of enzymatic systems for synthesizing this compound (3). It was found that after 4 hours the conversion of sedoheptulose-7-phosphate was comparable to that of the other substrates mentioned. It might be noted that at 2 hours sedoheptulose-7-phosphate was less extensively converted than these other compounds. The reason for this lag is not clear.

This conversion was similarly confirmed by showing that radioactive DHS was formed when radioactive sedoheptulose-7-phosphate was used as substrate together with inactive hexose diphosphate.

When hexose diphosphate and sedoheptulose-7-phosphate were

incubated together, the combination was about twice as efficient in forming DHS as was either compound when tested singly. This finding suggested a reaction involving both substrates; and one possible product of such a reaction might be sedoheptulose-1,7-diphosphate (SDP), which has recently been discovered as an enzymatic product by Horecker and Smyrniotis (1). SDP was therefore tested. It was found to be much the most efficient open-chain substrate used in forming DHS in this enzyme system, reaching a 30 per cent yield simply on incubation with the extract. The conversion of heptose phosphates to DHS is described in Table 3.

TABLE 3  
SYNTHESIS OF DHS FROM HEPTOSE PHOSPHATES

Substrate	$\mu M./ml.$	% Conversion (Molar)*	
		2 hrs.	4 hrs.
Hexose diphosphate	5	3.2	4.6
	2.5	3.2	3.6
Sedoheptulose-7-Phosphate	5	1.0	3.8
	2.5	1.5	4.5
Hexodiphosphate + Sedoheptulose-7-Phosphate	2.5 + 2.5	5.0	7.0
Sedoheptulose-1,7-Diphosphate	5	10	20
	2.5	15	30

\* Average values of several experiments. System same as in Table 1.

The synthesis of DHS ( $C_7H_{12}O_5$ ) from any carbohydrate,  $(CH_2O)_n$ , requires a net two-electron oxidation. Stoichiometric amounts of the pyridine nucleotides DPN and TPN were therefore incubated with SDP. The conversion of SDP to DHS was increased considerably in the presence of DPN, reaching values as high as 60 per cent; TPN had essentially no effect (Table 4). The considerable activity found in the absence of added DPN is presumably due to DPN in the crude extract, and possibly also to the activity of DPNH oxidase, which is known to be present in *E. coli* extracts (2). This interpretation was confirmed by treating an extract with charcoal to remove pyridine nucleotides. Eighty per cent of the



TABLE 4  
STIMULATION OF DHS SYNTHESIS BY DPN

SDP ( $\mu$ M./ml.)	DPN ( $\mu$ M./ml.)	TPN ( $\mu$ M./ml.)	% Conversion (Molar)	
			2 hrs.	4 hrs.
1.0	—	—	30	40
1.0	1.3	—	48	60
1.0	—	1.3	24	30

System same as in Table 1.

enzymatic activity was thereby eliminated, and addition of DPN fully restored the activity, i. e., to the level of 60 per cent conversion of SDP.

In summary, a soluble extract of a mutant strain of *E. coli* can form 5-dehydroshikimic acid from a number of phosphorylated sugars. It has been found that sedoheptulose-1,7-diphosphate is the most efficient substrate so far tested in this system. The conversion of SDP to DHS is markedly increased by the addition of DPN.

We would like to take this opportunity to thank Dr. B. L. Horecker of the National Institutes of Health for supplying ample quantities of all the labeled and unlabeled heptose phosphates, and the 6-phosphogluconic acid. This investigation would not have been possible without his generosity.

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## DISCUSSION

DR. VOGEL: I should first like to express my admiration for the fine study of aromatic biosynthesis by Dr. Davis and his many skillful collaborators. My further comments relate to that part of Dr. Davis' presentation which was not directly concerned with aromatic synthesis.

Dr. Davis suggested that the question is still open whether or not serine is a "normal" precursor of glycine in *Escherichia coli*. As evidence that glycine might be a "normal" serine precursor, Dr. Davis cited the existence of an *E. coli* mutant which gives a growth response to serine but not to glycine (on glucose-salt medium). However, evidence from tracer studies by Dr. Abelson shows that in wild-type *E. coli*, growing on glucose-salt medium, serine is indeed a precursor of glycine and not vice versa.

Dr. Simmonds has discussed tracer experiments with a mutant strain, such as Dr. Davis mentioned. The results obtained indicated that this mutant differs substantially from wild-type *E. coli* in aspects of serine metabolism. Therefore, Dr. Davis' conclusion on the basis of an unanalyzed growth response of a mutant of this type does not seem warranted. It is apparent that the notion of a "normal" precursor requires careful definition: the interesting experiments with fructose as a carbon source, as reported by Dr. Roberts, suggest that even major pathways involved in the serine-glycine relation of *E. coli* may vary with the main carbon source used.

Dr. Davis also reviewed the possibility that threonine is not a "normal" precursor of isoleucine, but can somehow give rise to the latter amino acid. As evidence against the "normal" function of threonine as isoleucine precursor, Dr. Davis mentioned the occurrence of one *E. coli* mutant (a), responding alternatively to several compounds including L-isoleucine, L-threonine, and D-threonine, and another (b) responding to all these compounds but not to L-threonine. Since the nature of the blocks in these mutants has not been established, the unanalyzed growth responses cited seem inadequate to resolve as delicate a metabolic question as "normal" versus "abnormal" precursors, especially since threonine and isoleucine synthesis are complicated by various types of inhibition and inhibition-relief phenomena. [The possibilities exist that mutant (a) has a partial deficiency in threonine synthesis, and that mutant (b) is blocked in isoleucine synthesis through unusual sensitivity to inhibition by threonine or a related metabolite; if these or similar interpretations are correct, the growth responses cited do not seem to help in evaluating the "normalcy" of threonine as isoleucine precursor.]

Strong, although not critical, support for the participation of threonine



in the direct path of isoleucine synthesis comes from the work of Dr. Abelson and collaborators. They found that  $C^{14}$ -threonine (less than 1 microgram per ml.), added to wild-type *E. coli* growing in  $C^{12}$ -glucose-salt medium, labels threonine and isoleucine (in the protein formed) at approximately equal specific activity.

Dr. Davis further suggested that results obtained with tracer methods might be less reliable than those obtained from enzyme studies. However, there appears to be no reason to believe that any given technique necessarily legislates over any other. In pathway studies, as in others, it seems likely that the best interpretations will be arrived at through a balanced evaluation of evidence from all available sources.

DR. DAVIS: In cases where two types of evidence lead to opposite conclusions it would be nice to know how to achieve this balanced evaluation; perhaps it would be more useful to tip the balance by attaching more weight to one kind of evidence than to the other.

DR. HOROWITZ: I would like to ask Dr. Davis whether parahydroxybenzoic acid and the unknown 6th factor have been found to be involved in the *Neurospora* mutants, as in *E. coli*?

DR. DAVIS: Not so far as I know.

DR. MCELROY: I would like to ask one question, which is how to come to a decision with respect to what kind of enzyme content you need in the cell before you get a certain amount of given product from the substrate. You commented here that you thought there was enough enzyme in the wild type to account for normal growth.

DR. DAVIS: From the concentration of a product in the cell and the generation time of that cell, one can readily calculate the rate required for the reactions leading to the synthesis of that product. With 5-dehydroquinase and with 5-dehydroshikimate reductase, this calculated rate per unit cell protein turned out to be of the same order of magnitude as the enzyme activity observed in wild-type extracts. I realize that the conditions found within the cell are not identical with the conditions that one finds to be optimal for enzyme activity outside the cell, and so I wouldn't want to lay any stress on the precision of the fit. It's just that the observed rates happen to have been of the right order of magnitude in these cases. If they had happened to be off I don't suppose it would have worried us.

DR. GUNSALUS: Incidentally, you may not be operating anywhere near the substrate saturation in the cell.

DR. DAVIS: Right.

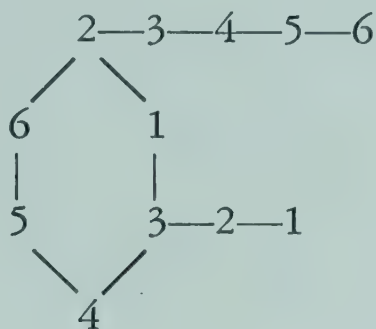
DR. NASON: Have you checked substrates such as steroids in terms of the dehydrogenase activity to see whether there are similar types of mechanisms with regard to reduction and oxidation?

DR. DAVIS: No. We haven't done any work on steroids. However, one might speculate about the possibility that the aromatization process in steroid metabolism resembles that observed with prephenic acid. It is of interest to note, for example, that non-aromatic steroid precursors of the aromatic estrogens have a methyl group that forms a quaternary carbon, and that this methyl group is eliminated in the process of aromatization.

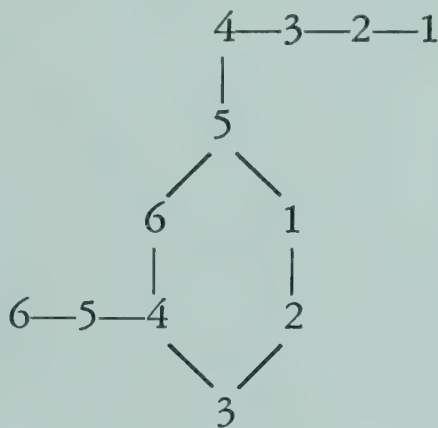
DR. BUSCH: I would like to ask whether Dr. Sprinson has considered the possibility of condensation of 2-6 units of a six carbon compound such as glucose or fructose; a 1-3 link and a 2-6 link might account for the isotope distribution that you have found.

DR. SPRINSON: Would you mind writing that down on the blackboard?

DR. BUSCH: This is what I mean:



DR. SPRINSON: Yes, we have considered such schemes. They do not explain all of the results. It would require at least one additional condensation to account for the following distribution of activity:



One is then left with a considerable fraction of activities which do not fit either of the two schemes, viz., the presence of activity from G-2 in S-5 and from G-5 in S-3.

DR. BUSCH: If the compound goes down through the glycolytic series and comes back up again you might get some labelling in the 5 position from the 2-carbon of glucose.

DR. SPRINSON: Any postulated involvement of glycolytic reactions to explain the behavior of carbon atoms 2 and 5 of glucose is faced with the difficulty that carbons 1 and 6 of glucose are not equilibrated with each other.



I should like to ask Dr. Gunsalus what he thinks of the possibility of a  $C_2$  plus  $C_1$  condensation (reversal of pyruvate decarboxylation) as an explanation for the conversion of acetate to glucose, when acetate is a sole carbon source.

DR. GUNSALUS: Direct reversal of the oxidative decarboxylation is very slight. The available data, of Korkes and of Goldberg and Sanadi, indicate only trace incorporation of  $C^{14}O_2$  during oxidative decarboxylation.

DR. NOVELLI: I would like to ask under what conditions were the experiments with C-1-labelled xylose done—that is, were they aerobic, or anaerobic?

DR. DAVIS: All of our experiments were aerobic.

DR. NOVELLI: I was talking particularly about the xylose experiments. I was worrying about how the xylose C-1 could go to the methyl of acetate. I would be curious why the methyl of acetate wouldn't go to the same position, but if it's aerobic that might tend to account for it.

DR. KALAN: Have you degraded the phosphoshikimic acid?

DR. SPRINSON: No. We hydrolyze the phosphoshikimate enzymatically, when it is known to be present, and isolate the total shikimate for degradation.

DR. WORK: I don't want to join issue with Dr. Davis, but this is relevant to Dr. McElroy's question. We have calculated that there really isn't enough diaminopimelic acid decarboxylase in the cell to provide all lysine requirements. Then the other point which was brought up by Dr. Davis about this apparent lack of incorporation of exogenous diaminopimelic into *E. coli*—it is interesting. We did have a look at the strain of *E. coli* used by the Carnegie workers and Dr. Hoare found that fresh cells are extremely impermeable to both lysine and diaminopimelic acid. Maybe I shouldn't say "impermeable," but at least these amino acids don't easily reach the sites of their decarboxylases. The evidence for this was the difficulty in getting decarboxylation of diaminopimelic acid or lysine until the cells were dried. Normally with *E. coli*, acetone-drying produces about a doubling of the lysine decarboxylase and possibly tripling of the diaminopimelic decarboxylase, whereas in this particular strain of *E. coli* the lysine decarboxylase activity increased 10-fold upon drying from 30 to 300. The diaminopimelic decarboxylase was hardly detectable at all in fresh cells and showed a  $Q_{CO_2}$  of 9 in acetone-dried cells. So it does look as if there is an unusually big permeability barrier in this strain between the external medium and the enzyme sites.

DR. McELROY: Would Dr. Kalan care to comment further on the mechanism of cyclization of sedoheptulose?

DR. KALAN: The efficient enzymatic conversion of sedoheptulose diphosphate to dehydroshikimic acid suggests that the diphosphate might be utilized without breakdown of its carbon chain. On the other hand, the known

enzymatic reaction by which sedoheptulose diphosphate is formed from glucose, followed by cyclization of its chain, would lead to an isotopic distribution of shikimic acid very different from that presented by Dr. Sprinson. It therefore appears either that sedoheptulose diphosphate is formed in the intact cell by routes that have not yet been revealed by the studies performed so far on extracts, or that the sedoheptulose diphosphate chain undergoes disruption before it is cyclized. This question is now under investigation.

DR. MACKENZIE: I would like to suggest with reference to alternative pathways of metabolism that different people like to eat different things. No doubt there are major pathways *on a given medium* but the implication that a given environment (medium) is the 'normal,' 'best' or 'usual' one is a dangerous supposition that reflects the bias of the experimenter without adequate consideration of the bacteria and its fluctuating environment.

DR. HANDLER: I would like to be brief and perhaps impertinent, if I may, with respect to Dr. Davis' reference to this dictum that we have been hearing about—the advantages of studying mammalian metabolism in *Neurospora* rather than in *E. coli*. I am reminded of a little story. A gentleman, highly dissatisfied with the chicken salad that he had been served at a particular restaurant, complained to the waiter. The waiter looked at him somewhat shocked, and said, "Why, sir, we make our chicken salad with the best pork, and that makes better chicken salad than any tuna fish you ever saw." Some of us still prefer chicken in our chicken salad!



# THE METABOLISM OF PHENYLALANINE AND TYROSINE

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MOST OF THE phenylalanine and tyrosine in the animal body is eventually converted to acetoacetate, in keeping with the role of these compounds as ketogenic amino acids. Only this aspect of their metabolism will be discussed here. The quantitatively less important routes, to DOPA and melanin, to adrenalin, and to thyroxine, have been discussed in recent reviews (2, 16, 45, 46, 66, 67).

## THE PROTOTYPIC STUDY OF A METABOLIC PATHWAY

The history of phenylalanine and tyrosine metabolism is coincident with the study of that hereditary condition in man called alcaptonuria. The homogentisate formed from these amino acids and excreted by alcaptonuric patients was the first nitrogen-free metabolic fragment of an amino acid to be recognized. The experimental opportunities and challenges offered by this disease attracted the early investigators in the new field of biochemistry, who soon showed remarkable progress. As soon as the structure of homogentisic acid was determined, in 1891, the role of tyrosine as its precursor was recognized (93). "Exogenous" and "endogenous" metabolism were very soon seen to have no meaning here, since homogentisate was formed indiscriminately from the appropriate amino acids whether from the diet or from tissue breakdown. But the most striking result of these early studies was the clear formulation by Garrod, before 1907, of the concept now called biochemical genetics (29). The interrupted phenylalanine and tyrosine metabolism of the alcapto-

nurics was the first to be recognized of his famous "inborn errors of metabolism"—the hereditary lack of a usual metabolic reaction.

With the single mutant of alcaptonuria available, and with the assumptions currently used in the genetic analysis of a metabolic pathway, the probable intermediates between phenylalanine and homogentisate were established in Germany well before the first World War (57, 58). Subsequently, all possible techniques have been utilized as they became available to confirm or define more exactly the pathway as originally determined. The history of phenylalanine and tyrosine metabolism therefore presents the prototype of the genetic analysis of a metabolic pathway: a pathway now sufficiently well studied and well understood to demonstrate the roles played by each of the several techniques available for investigation of these phenomena.

#### NEUBAUER'S SCHEME, 1909

The pathway from phenylalanine to homogentisate deduced from experiments with alcaptonurics is reproduced in Fig. 1, as given by Neubauer (59). Other experiments had established acetoacetate to be an eventual product beyond homogentisate (4, 17). Today we can demonstrate the reactions connecting this same sequence of intermediates (cf. Fig. 12). The conversion of phenylalanine to tyrosine is direct, and not by way of the keto acids. Neubauer's use of solid and dotted arrows indicated the greater likelihood of the direct conversion. The two routes shown were equally possible between tyrosine and 2,5-dihydroxyphenylpyruvate, the immediate precursor of homogentisate. The order of the reactions, whether tyrosine was first deaminated to *p*-hydroxyphenylpyruvate and then oxidized in the ring, or first oxidized in the ring to form 2,5-dihydroxyphenylalanine and then deaminated, was not known. 2,5-Dihydroxyphenylalanine had not yet been tested or even synthesized. *p*-Hydroxyphenylpyruvate (*p*HPP) had been tested, however, and like all the other intermediates did form homogentisate in the alcaptonuric person. Recently Neuberger synthesized 2,5-dihydroxyphenylalanine and tested it in an alcaptonuric (61). It too was



converted to homogentisate. The alternative routes sketched by Neubauer therefore remained equally possible until more recent studies on the enzyme reactions themselves decided which was the actual route from tyrosine (through *p*HPP, see below).

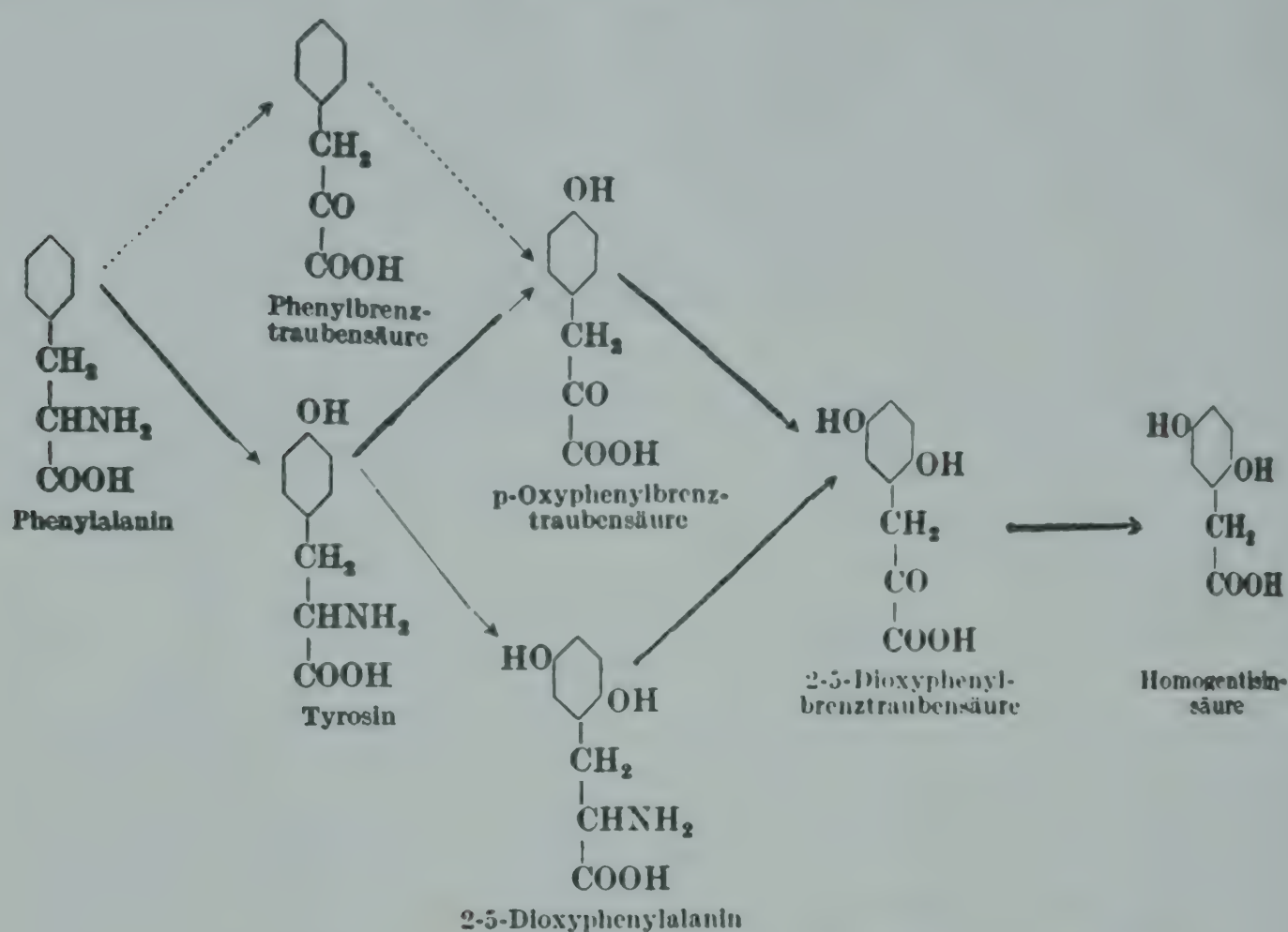


FIG. 1. Neubauer's scheme of the pathway for phenylalanine metabolism, deduced from experiments on alcaptonurics (59).

### MIGRATION OF THE SIDE-CHAIN

Only one objection, but a very fundamental one, was raised against the Neubauer scheme of phenylalanine metabolism. It was objected that the pathway was in a sense an artifact, an abnormal set of reactions leading to an abnormal metabolite seen only in alcaptonurics, and that the pathway did not represent the *normal* route of this metabolism. The objection was really a challenge to biochemical genetics, which only later could prove itself as a method. The preliminary skirmish on this broad question was fought over the peculiar conversion of a 4-hydroxyphenyl- to a 2,5-dihydroxyphenyl-compound to give homogentisate, the pivotal intermediate of the

Neubauer scheme. Migration of the side-chain or migration of an hydroxyl group had to be assumed to form homogentisate from tyrosine, and this was the "abnormal" reaction of the scheme. It seemed so unlikely even to its proponents that they at first attributed this conversion to the bacteria of the gut. [The reaction ". . . erschien Baumann so erstaunlich, dass er glaubte, eine solche Veränderung auf Bakterientätigkeit zurückführen zu müssen" (59, p. 860).] But

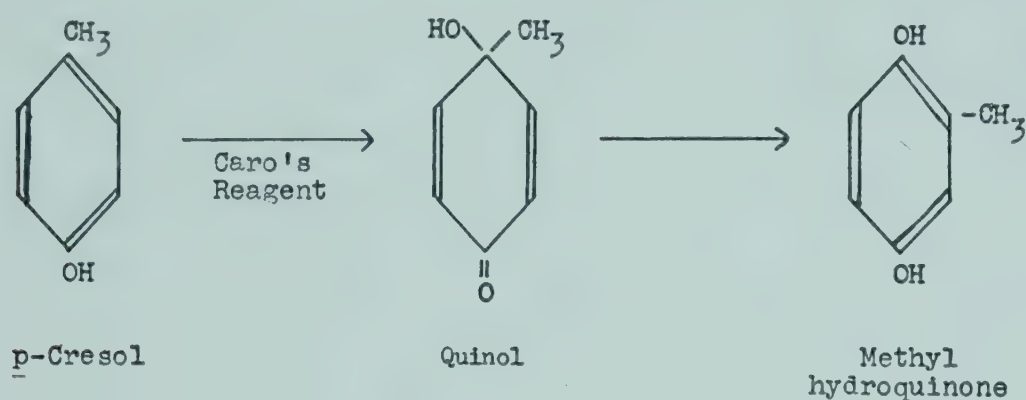


FIG. 2. The migration of a methyl group during oxidation of *p*-cresol (6). The intermediary quinol provided the basis for the analogous compound postulated as the biologic precursor of homogentisate (27).

when a similar transformation of *p*-cresol into methylhydroquinone was demonstrated in organic chemistry (5, 6), the reaction was readmitted to the metabolism of man from its bacterial limbo. A quinol intermediate, analogous to that formed in the oxidation of *p*-cresol (Fig. 2), was postulated as the metabolic precursor of homogentisate (27, 50). Recently Witkop made this quinol from *p*-hydroxyphenyl acetate and found it inactive in a liver system oxidizing tyrosine (92). It is now clear that the intramolecular rearrangement must occur before 2,5-dihydroxyphenylpyruvate, as was postulated by Neubauer, and the correct quinol intermediate must have a pyruvate instead of an acetate side-chain. A quinol with a pyruvate side-chain has not yet been made, nor is there direct evidence that one occurs in biological systems (85). Nevertheless, the distribution of isotopes in acetoacetate formed from labelled phenylalanine and tyrosine (Fig. 3) has settled the fact that an intramolecular migration of the side-chain on the ring occurred at some stage in the metabolism of these compounds (88, 74). The mechanism and intermediates of this migration still remain to be demonstrated.



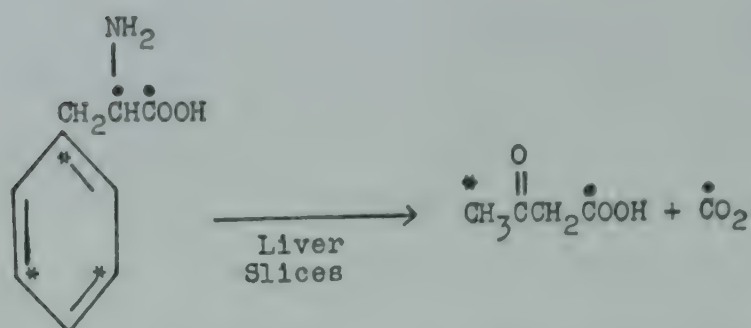


FIG. 3. The metabolism of doubly labelled phenylalanine, according to Schepartz and Gurin (74). Between the differently labeled carbons in the acetoacetate were two unlabelled carbons instead of one as in the original phenylalanine, which proved the intermediary migration of the side-chain, probably to an *ortho* position.

### AN ALTERNATIVE OR ABNORMAL PATHWAY

The apparently reasonable hypothesis of a quinol intermediate was tested by Dakin with results which can only be described as the *reductio ad absurdum* of this hypothesis and which seriously challenged the relevance of the Neubauer scheme to normal metabolism.

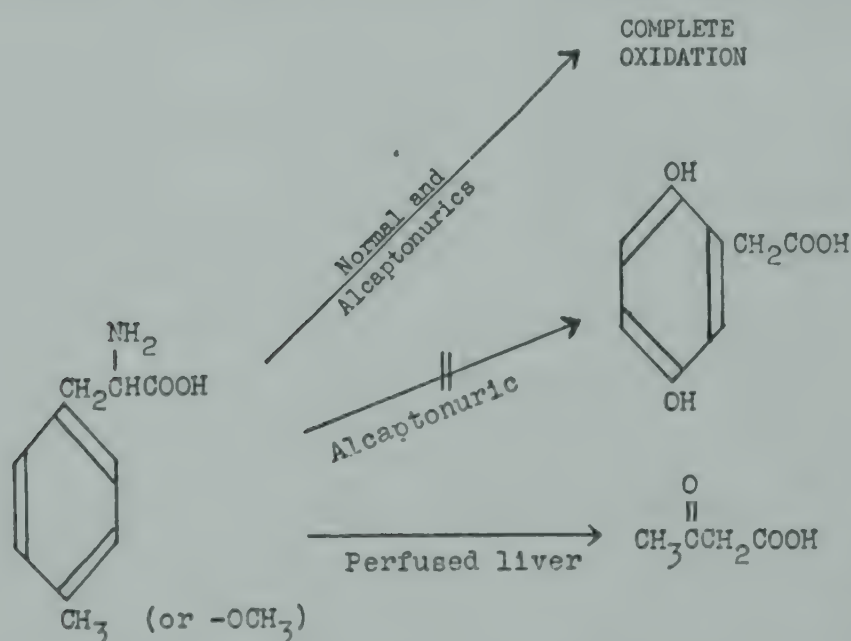


FIG. 4. Diagrammatic summary of the metabolic fate of phenylalanine analogues which would not form the postulated intermediary quinol. Such compounds were nevertheless oxidized, giving rise in liver to acetoacetate, and did not form homogentisate in alcaptonurics (15).

The paradox created has been left unresolved. Dakin took the view that if Neubauer's scheme were the pathway for the metabolism of aromatic compounds, then related compounds which could not form an intermediate quinol should not be oxidizable. *p*-Methylphenylalanine and *p*-methoxyphenylalanine (Fig. 4) could not form

quinols. But these compounds, and others (28), were nevertheless oxidized by normal animals, and in liver gave rise to acetoacetate just as did tyrosine. Even more disturbing was the finding that in alcaptonurics these compounds were completely oxidized without formation of any homogentisic acid (14, 15). The conclusion appeared inescapable that the metabolism of these compounds occurred by another route, perhaps the normal route, which did not involve a quinol or homogentisate but which did end in acetoacetate formation. A different type of evidence leading to a similar conclusion has been provided by Katsch (35). The conversion of tyrosine to homogentisate in alcaptonurics ceased in ketosis, which could be explained if an alternative metabolic route were available (or if the genetically absent homogentisate oxidation could be activated under certain conditions).

Gradually over the subsequent years various experiments restored confidence in the Neubauer scheme as a normal pathway, although it lacked any direct proof until recently. During the same time no work lessened the likelihood of Dakin's alternative route. At first every effort had been made to detect homogentisate formation in normal animals and so to regularize the pathway deduced from alcaptonuric metabolism. Experimental (i. e. non-genetic) alcaptonuria was eventually produced, by heroic doses of tyrosine or phenylalanine (1, 64, 26), protein deficiency (62), and by  $\alpha,\alpha$ -dipyridyl treatment (83). But the necessary conditions were too abnormal in themselves to indicate that homogentisate was an intermediate of *normal* metabolism. It was established, however, that under certain conditions animals lacking the particular genetic trait could make homogentisate.

Discoveries unrelated to this line of inquiry did still more to bolster the Neubauer scheme. A new hereditary metabolic error, phenylketonuria (25), was in time recognized to be a block in the direct conversion of phenylalanine to tyrosine (33). The latter reaction was now well-established from nutritional (94) and from isotopic (54) experiments. Another intermediate, *p*HPP, was excreted after phenylalanine or tyrosine administration by a single patient,



described as a case of "tyrosinosis" by Medes (48). Later the same intermediate was found in the urine in scurvy: in scorbutic guinea pigs (78), premature infants (47), and scorbutic infants (53), adult persons (72), and monkeys (73). Homogentisate was also found, but only in one set of experiments (78), and not in any others. Its presence then can be attributed to the large dose of tyrosine given (cf. 64) and not to a second metabolic block in scurvy, as is sometimes assumed. A second vitamin was apparently involved in the metabolism of tyrosine when large doses of pteroylglutamic acid were found to lessen the excretion of *p*HPP by scorbutic guinea pigs (95, 96). Lerner's suggestion that Medes' single case of tyrosinosis may have been due to unrecognized scurvy (45), since both states were characterized by *p*HPP excretion, was no sooner made than two more cases were reported (23), in neither of which did ascorbic acid lessen the excretion of *p*HPP after tyrosine dosage. At least two means of accumulating this postulated intermediate therefore existed. The compounds suggested by Neubauer were thus proving to be physiologically important, even if they could not yet be related sequentially in normal metabolism as he had postulated.

#### THE PROBLEMS FOR ENZYMATIC ANALYSIS

Very few of the reactions in the normal metabolism of phenylalanine and tyrosine had actually been established by the work on intact systems. The conversion of phenylalanine to tyrosine, and the over-all conversion of phenylalanine to acetoacetate (with intermediate rearrangement of the side-chain on the ring) were known to occur. The conversions of tyrosine to *p*HPP and to homogentisate were familiar under abnormal conditions, as was the conversion of the alternative intermediate, 2,5-dihydroxyphenylalanine, to homogentisate. But under other conditions, notably in the experiments of Dakin with analogues, the reactions apparently followed a quite different route. It was therefore necessary to determine the actual reactions of tyrosine to each of its intermediates in turn. There was also a growing need to understand the individual reactions them-



selves: to settle whether a hereditary metabolic error was due to absence of the particular enzyme, and to decide if the metabolic derangement in scurvy was due to a direct, coenzyme-like action of the vitamin on an enzyme. The study of the enzyme reactions making up phenylalanine and tyrosine metabolism, which alone could give the necessary information, will be discussed in the order of the reactions starting from phenylalanine.

### L-PHENYLALANINE OXIDATION

The oxidation of phenylalanine to tyrosine is a direct reaction, as previously mentioned. But, as we are finding with many of the enzymes acting upon aromatic compounds, the enzyme responsible for phenylalanine oxidation is different from any we have yet known (87, 51). This enzyme will be described later by Udenfriend. Here it need only be recorded that after this reaction had been demonstrated in the livers of normal animals and man, an opportunity was seized by Jervis to test for its presence in the livers of two phenylketonurics (34). Its absence in the livers of these patients provided the first experimental basis for the enzymelessness of an "inborn error of metabolism" in man, and directly supported the one-gene-one-enzyme concept upon which had rested so much inference. The possible improvement in the condition of a phenylketonuric child given a low phenylalanine diet (8), if substantiated, would provide the first indication that the metabolic block affects the mind through the accumulation of something rather than by causing a deficiency of something. The *o*-hydroxyphenyl compounds identified recently in the urine of such patients (9) have not yet been fitted into the picture, either as toxic substances, or as normal metabolic intermediates.

### THE OVER-ALL L-TYROSINE OXIDATION IN VITRO

*Crude Liver Breis.* The first oxidation of L-tyrosine to acetoacetate in crude liver breis, by Bernheim and Bernheim in 1934, established almost all that has been learned with certainty about the reaction in



this type of preparation. They found four atoms of  $O_2$  consumed per molecule of tyrosine. No ammonia was produced (7). One acetoacetate, one or two molecules of  $CO_2$ , alanine (20), and malate (44) were later recognized as products. Concentrated preparations, with correspondingly high blanks, had to be used to avoid the great dilution effects in the system (7, 3, 79). There was uncertainty in all the work about any quantitative measurements intended to reveal

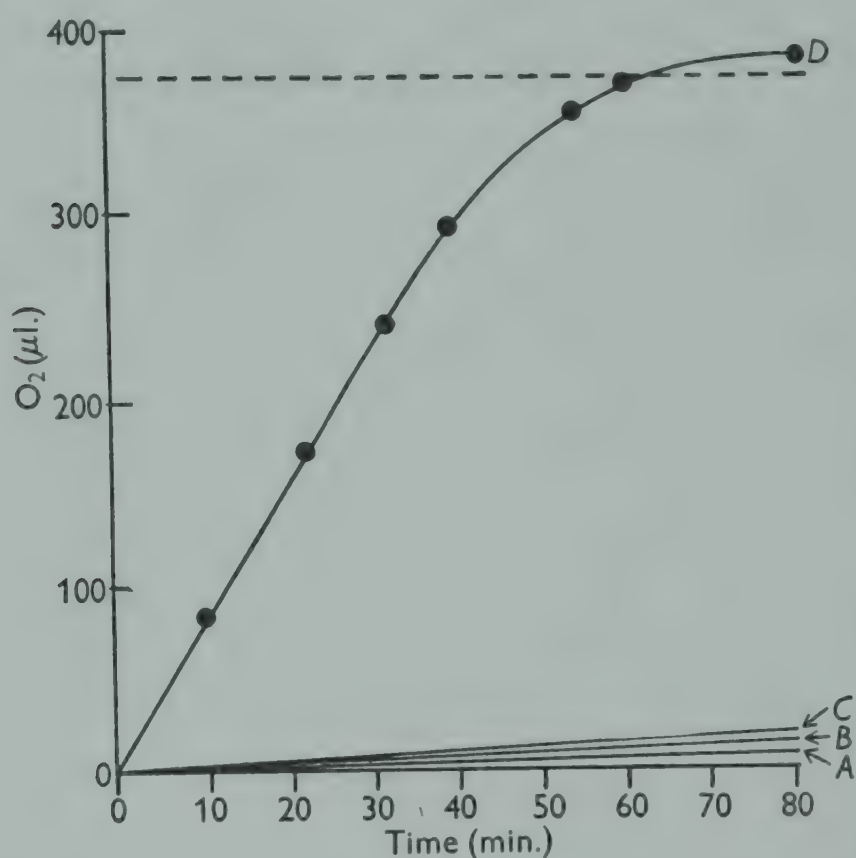


FIG. 5. The requirement for  $\alpha$ -ketoglutarate in the oxidation of L-tyrosine by a soluble, dialyzed liver preparation (39). All experiments contained enzyme and ascorbic acid. A, no further additions; B, plus tyrosine; C, plus  $\alpha$ -ketoglutarate; D, plus tyrosine and  $\alpha$ -ketoglutarate. The dotted line indicates the theoretical oxygen consumption, 4 atoms per molecule of tyrosine, for the conversion to fumarate, acetoacetate, and  $CO_2$ .

the intermediate reactions, and difficulties were experienced in getting the reaction to go to completion. The school of Felix carried out the most thorough studies on the crude system (19, 97, 20, 21, 22), and its uncertainties led them with apparently good reason to abandon *p*HPP and homogentisate as possible intermediates of tyrosine oxidation. Their conclusions complicated the dilemma enmeshing the field by indicating a metabolic pathway quite different from any compatible with experimentation on the intact animal (22). Attempts

to test the effects of ascorbic or pteroylglutamic acids in the reaction of the crude liver brei also gave small differences or inconclusive results (70, 71, 69, 63).

*Reinforced Liver System.* The difficulties of the crude liver brei reaction disappeared when the needed two cofactors for the over-all oxidation of tyrosine were recognized (43). Active, soluble systems with low blanks could then be obtained. These systems carried out the over-all reactions deduced earlier by Neubauer, and with such systems those points of uncertainty left by the intact animal experiments could be decided. The two cofactors needed were ascorbic acid and  $\alpha$ -ketoglutaric acid. The latter was required (Fig. 5) for an initial obligatory transamination of tyrosine preliminary to its oxidation. This step was recognized in 1950, the *annus mirabilis* of new transaminations (18, 10, 31, 81).

#### TYROSINE TRANSAMINATION

The transamination step was identified by the formation of glutamate from  $\alpha$ -ketoglutarate as the L-tyrosine was oxidized and, when oxidation was prevented by anaerobiosis or by absence of ascorbic acid, by the accumulation of *p*HPP from the tyrosine. Other experiments showed that the initial transamination of tyrosine was an essential step in the oxidation reaction (39). The transaminase could be partially resolved, while retaining the oxidative activities of the system, and this caused a parallel slowing of both the transamination and the oxidation of tyrosine. Addition of the transaminase coenzyme, pyridoxal phosphate, increased the rates of both the transamination and the subsequent oxidation, which was therefore dependent upon the initial transamination (Fig. 6).

An initial transamination to *p*HPP during tyrosine oxidation was promptly thereafter also shown by Schepartz (75, 76), on the basis of a requirement for  $\alpha$ -ketoglutarate for oxidation to occur and the associated formation of glutamate. LaDu and Greenberg (41) subsequently also reported an activating effect of  $\alpha$ -ketoglutarate (and of arsenite, and inhibition by pyruvate) on tyrosine oxidation,



for which they suggested that a transamination might be the most likely explanation.

The specificity of the tyrosine transaminase toward other aromatic amino acids and toward the keto-acid acceptors,  $\alpha$ -ketoglutarate and possibly pyruvate, deserves further study. Felix et al. (24) con-

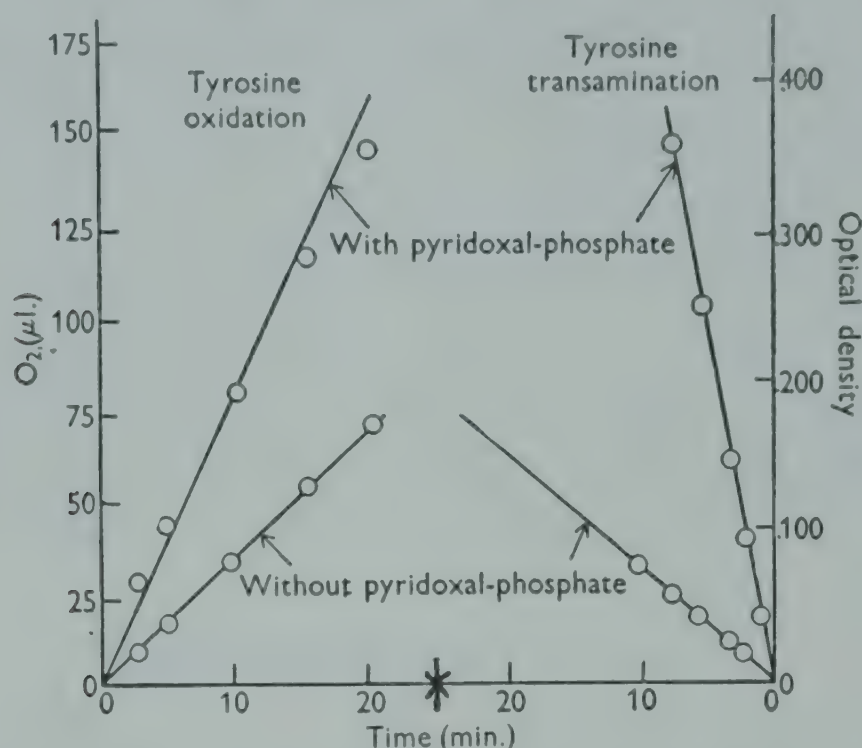


FIG. 6. The dependence of the rate of L-tyrosine oxidation upon the rate of tyrosine transamination in a partially resolved enzyme system (39). Addition of pyridoxal phosphate increased the rates both of transamination (right) and oxidation (left) measured separately in the same enzyme preparations.

firmed the occurrence of the transamination step with  $\alpha$ -ketoglutarate, but suggested that alternatively pyruvate might participate directly in the transamination to give rise to the alanine they had earlier found. A subsequent glutamate-alanine transamination could also account for this accumulated alanine (39). Felix et al. made the interesting suggestion that instead of a usual transamination of tyrosine to its keto acid, a definitive reaction with loss of the amino group, shift of the side-chain, ring oxidation, and oxidative decarboxylation to form homogentisate might all happen to the intermediary transamination complex itself. This suggestion emphasizes how little we still know for certain about these strange reactions. However, the apparent near-simultaneity of glutamate formation, O<sub>2</sub> uptake, and CO<sub>2</sub> evolution measured during active tyrosine oxidation does not exclude the occurrence of separate, sequential reactions.

Moreover, the formation of at least one intermediate, *p*HPP, is well established.

p-HYDROXYPHENYLPYRUVATE AS AN INTERMEDIATE  
IN L-TYROSINE OXIDATION

The studies of Neuberger completed the proof that the two alternative routes from tyrosine, involving the order of occurrence of side-chain deamination and ring-oxidation that were given in the Neubauer scheme, were both equally possible. The formation of *p*HPP from tyrosine in several situations could possibly be interpreted as lending support to this compound as the true intermediate, especially since the conversion of tyrosine to 2,5-dihydroxyphenylalanine had not been shown. The latter reaction was definitely excluded when no oxidation of tyrosine, not even the one atom for ring oxidation, occurred in the absence of the  $\alpha$ -ketoglutarate necessary for the initial side-chain deamination (see Fig. 5). A direct test of the postulated product of a primary ring oxidation, 2,5-dihydroxyphenylalanine, showed it to be virtually inactive in the soluble system which oxidized tyrosine (39). In crude liver brei it was subsequently found to react, probably with another transaminase, and this reaction, through 2,5-dihydroxyphenylpyruvate, could account for the observed conversion of 2,5-dihydroxyphenylalanine to homogentisate in alcaptonurics. On the other hand, the three positive pieces of evidence obtained with the soluble enzyme system for *p*HPP as the true intermediate of tyrosine oxidation were (1) its formation from tyrosine; (2) the effects of pyridoxal phosphate on its formation and of ascorbic acid on its removal, paralleled by the appropriate effects of these substances on the over-all oxidation of tyrosine; and (3) the oxidation of *p*HPP at a similar rate and to the same final product as tyrosine in the soluble system. An apparent difference, due to the fact that the system needed more ascorbic acid to oxidize *p*HPP than to oxidize tyrosine, will be described later.



## THE ASCORBIC ACID REQUIREMENT

The clear demonstration of transamination in tyrosine oxidation was possible in part because the need for ascorbic acid was recognized at the same time, and it was added to all the reactions in Fig. 5. In Fig. 7  $\alpha$ -ketoglutarate was added to all the reactions, and ascorbic acid was omitted from two of them. It can be seen that tyrosine oxidation shortly stopped in the absence of ascorbic acid.

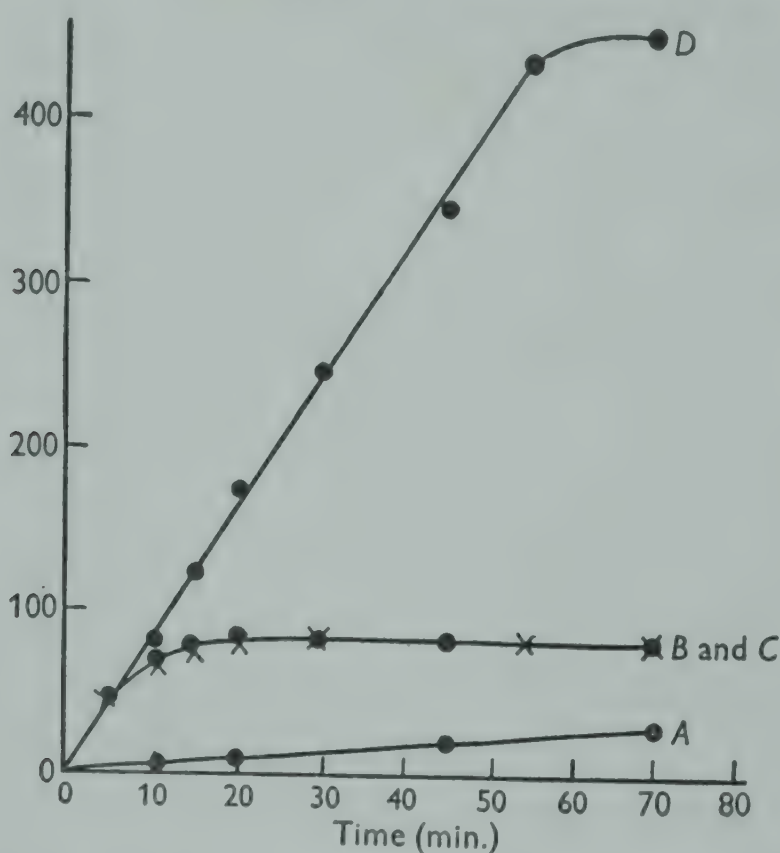


FIG. 7. The effect of ascorbic acid on the oxidation of L-tyrosine (39). All reactions contained  $\alpha$ -ketoglutarate and the soluble, dialyzed liver enzyme. A, ascorbic acid; B and C, tyrosine alone and plus glutathione, respectively; D, ascorbic acid and tyrosine.

It could not be replaced by glutathione, or a number of other substances. The close analogue of ascorbic acid, isoascorbic acid, was found to be equally effective in replacing ascorbic acid. From many experiments with these favorable enzyme preparations, it was clear that the amount of ascorbic acid needed was less than the amount of tyrosine oxidized (i. e., catalytic amounts of ascorbic acid were used). The small amount of oxidation which occurred in the absence of ascorbic acid was due to leakage through the blocked first oxida-

tion step (of *p*HPP). In contrast to the usual behavior of a coenzyme was the unusual feature that suboptimal amounts of ascorbic acid permitted the reaction to go at nearly the optimal initial rate, but let it stop sooner.

There is little doubt that the effect in vivo of ascorbic acid on the oxidation of *p*HPP is part of its vitamin C action. While rather higher than antiscorbutic doses of ascorbic acid have been necessary to insure the normal metabolism of high doses of tyrosine, the antiscorbutic amounts are apparently adequate for the amounts of tyrosine normally metabolized (39). (It will be noted below, in keeping with this view, that the ascorbic acid requirement of the enzyme system in vitro is proportional to the *p*HPP concentration present). Isoascorbic acid, which has only about one-twentieth the antiscorbutic action of ascorbic acid, also has about one-twentieth of ascorbic acid's effect on the tyrosine metabolism in guinea pigs (78).

Because the system in vitro required the addition of ascorbic acid even when prepared from normal animals, it provided an assay in vitro of a physiological effect of vitamin C. This assay revealed isoascorbic acid to be equally as active as ascorbic acid. The same was subsequently shown for glucoascorbic acid (80, 42, cf. 63). These results indicated equal potential vitamin C activities in these compounds, at least in relation to tyrosine metabolism, and the lower activities in vivo of the analogues could be attributed to their poor retention in the body (39).

#### SPECIFICITY OF THE ASCORBIC ACID ACTIVATION OF *p*HPP OXIDATION

Particular interest has centered on the oxidation of *p*HPP, as to how the ring oxidation and the side-chain migration occur, and where ascorbic acid exerts its effect. The main emphasis to date has been directed toward the nature of the action of ascorbic acid, particularly as revealed by the ability of certain other compounds to replace it in the system catalyzing the over-all reaction. Table 1 lists the types of compounds which have been tried in the activation of tyrosine oxidation. The first two columns, marked off by a double



TABLE 1  
ACTIVATION OF L-TYROSINE OXIDATION

	Rienits	Painter & Zilva	Knox & Knox	Sealock et al.	LaDu & Greenberg
Ascorbic acid	+	+	+	+	+
Isoascorbic acid	—		+	+	+
Glucoascorbic acid	—	+		+	+
Dichlorophenolindophenol					+
Hydroquinone					+
Reductone				+	
Dihydroxymaleic acid			—		—
Glutathione			—	—	—
Cysteine			—		—
H <sub>2</sub> O <sub>2</sub>			—		
Catechol					—
DOPA					—
2-Methyl ascorbic acid				—	
2,3-Dimethyl ascorbic acid				—	
Pteroylglutamic acid	+		—		

Summary of the types of compounds tested for ability to replace ascorbic acid in the oxidation of L-tyrosine by cell-free liver preparations. +, effective; —, ineffective; blank, not tested. (Ref. 69, 63, 39, 80, 42).

line, are experiments, done with crude liver breis, in which only small effects could be observed. The subsequent columns refer to experiments with the reinforced system described above, and using either soluble dialyzed liver fractions (third column) or liver acetone powder extracts (last two columns). Activation of tyrosine oxidation in the liver enzyme system was not restricted to ene-diol compounds: Certain other easily oxidized compounds like dichlorophenolindophenol and hydroquinone also showed this effect in the system tested (42). But considerable oxidation of tyrosine occurred in the acetone powder preparations without any addition of ascorbic acid. As LaDu and Greenberg pointed out, the activations observed by them with compounds other than ascorbic acid could possibly be attributed to their protection of residual ascorbic acid in the system. Still, other similar compounds were inactive, as was H<sub>2</sub>O<sub>2</sub>, which might be a

common product of the easily oxidized compounds. The effect reported for pteroylglutamic acid in the crude liver brei (69) could not be demonstrated in the more active reinforced system (39). Tyrosine oxidation in crude liver breis from pteroylglutamic-acid-deficient rats was reported to be activated in vitro by pteroylglutamic acid (70, 71).

#### CORRESPONDENCE OF THE OVER-ALL TYROSINE REACTION IN VITRO WITH THE POSTULATED PATHWAY IN VIVO

By good fortune all the reactions of the over-all oxidation of tyrosine to acetoacetate occurred in the same soluble fraction of liver, so that this single preparation reproduced the known reaction of tyrosine to acetoacetate in vivo. Analysis of the reaction in vitro also confirmed the pathway postulated by Neubauer. L-Tyrosine was oxidized upon the addition of both  $\alpha$ -ketoglutarate and ascorbic acid. *p*HPP, identified as an intermediate above, was oxidized with the addition of only ascorbic acid. The action of ascorbic acid at the *p*HPP oxidation step was indicated by its not being required for the oxidation of the subsequent intermediates. The other postulated intermediates of Neubauer, 2,5-dihydroxyphenylpyruvate and homogentisate, but not 2,5-dihydroxyphenylalanine, were oxidized by the same system, at appropriate rates and to the same product as tyrosine. The intermediates consumed the theoretical amount of oxygen calculated for them as intermediates in the oxidation of tyrosine. A recent report from Japan has claimed the demonstration by chromatography of 2,5-dihydroxyphenylpyruvate accumulated from *p*HPP in a slow-acting liver enzyme system (86). This result is to be expected to complete the proof, if the former keto acid is indeed an intermediate.

*Identification of Homogentisate as a Normal Intermediate.* The pivotal compound, homogentisate, was accumulated from tyrosine in vitro (39). Acid treatment of the soluble liver preparation inactivated only the homogentisate oxidase and at the same time stopped the oxidation of tyrosine at half the usual oxygen uptake.



Homogentisate then accumulated during the oxidation of L-tyrosine and was isolated and identified chromatographically. This demonstration finally fixed homogentisate as a normal intermediate, not an abnormal metabolite seen only in alcaptonuria and certain abnormal states, and established the relevance of the Neubauer scheme to normal tyrosine metabolism.

#### INDIVIDUAL ENZYME REACTIONS OF TYROSINE OXIDATION

The liver system in vitro so far described provided an intact series of reactions which could be identified with those thought to occur in the intact animal and in which the effect of ascorbic acid could be demonstrated. But the further identification of the separate reactions, including the inevitable minor intermediates undetectable in the intact animal experimentation, required taking apart the over-all system of enzymes that convert L-tyrosine to acetoacetate. At present we have only a beginning acquaintance with the oxidation of *p*HPP, but a fairly complete knowledge of homogentisate oxidation.

*Oxidation of p*HPP. The single oxidation step, *p*HPP to 2,5-dihydroxyphenylpyruvate, has not yet been obtained as an effective reaction. The simplest reaction so far studied was that of *p*HPP to homogentisate, carried out by the acid-treated enzyme (39), or obtained by inhibition of homogentisate oxidation with  $\alpha,\alpha$ -dipyridyl (LaDu, unpubl.).

The activation by the non-ascorbic acid compounds, presumably of the *p*HPP step, was determined with the over-all oxidation of tyrosine. In addition to the possibility already mentioned, that the activations could have been due to preservation of some residual ascorbic acid, there has been no direct proof that the activation observed was at the *p*HPP oxidation step. Williams and Srinivasan (89, 90) have indeed suggested that dichlorophenolindophenol (DCPP) activates at a later locus than does ascorbic acid. We have reinvestigated these matters, although not with the isolated enzyme for the *p*HPP oxidation step, as we would like to have done.

The uncertainties growing out of the occurrence of the several reactions have been minimized by (1) the use of systems showing virtually no oxidation in the absence of ascorbic acid; (2) the use of *p*HPP itself as substrate, in order to avoid limitations of transamination; and (3) the chemical determination of *p*HPP disappearance (the Millon reaction) as a measure of the oxidation step in

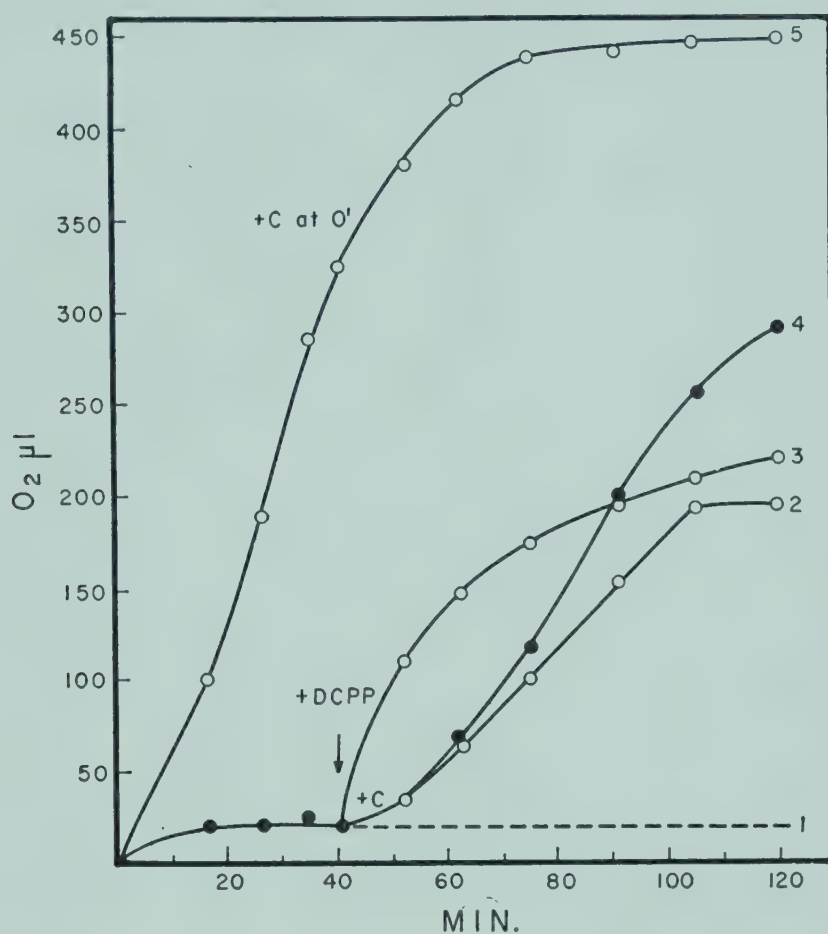


FIG. 8. The activation of L-tyrosine oxidation at the *p*HPP oxidation step by ascorbic acid (C) or by dichlorophenolindophenol (DCPP). The effect of 0.5 mg. ascorbic acid added initially (5) compared with the 2.0 mg. and 4.0 mg. that were required when added after 40 minutes (2 and 4, respectively) indicates the greater ascorbic acid requirement after *p*HPP had accumulated in the system. The effectiveness of 1.25  $\mu$ M. DCPP (3), when there was no blank oxidation and when only relatively large amounts of ascorbic acid were effective in starting the oxidation, demonstrated that the effect of DCPP was not one of preserving any residual ascorbic acid in the system (Gregerman and Knox, unpub.).

question instead of the total oxygen uptake (30). With such systems it was possible to prove that DCPP and hydroquinone did replace ascorbic acid for the *p*HPP oxidation reaction itself under conditions in which any preservation of residual ascorbic acid would have been ineffective (Fig. 8).



While using *p*HPP routinely as the substrate, it was noted that more ascorbic acid was required than was needed for the same enzyme to oxidize tyrosine. The reason for this fact is not yet clear, but it has explained several puzzling properties of the system. Since the concentration of *p*HPP present during tyrosine oxidation, and therefore the ascorbic acid requirement, was determined by the rate of its generation in transamination and its removal by oxidation, the different balances of the component reactions in different enzyme preparations can account for the observed variations in the optimal ascorbic acid requirements. The inhibition caused by *p*HPP accumulation in the presence of a given suboptimal amount of ascorbic acid also accounted for the oxidation under these conditions at an apparently optimal rate, but only for a short time. A similar explanation accounted for the observation of Williams and Srinivasan that DCPP still activated the oxidation of tyrosine when added some time after the incubation had begun, but that ascorbic acid acted only if it was added at the beginning of the incubation of enzyme and tyrosine. This was the basis of their conclusion about an action of DCPP at a later step. If *sufficient* ascorbic acid was added later to insure oxidation of the *p*HPP which had built up during the previous incubation, it activated as usual (Fig. 8). DCPP is effective in smaller amounts.

The lower oxygen uptakes of the reactions activated later (in Fig. 8) also illustrate the danger of using the oxygen uptake of the over-all oxidation as a measure of the first reaction. Due to the fragility of the subsequent oxidative reactions the total oxygen uptake was less in the later-activated reactions than in the one activated from the beginning. Measurement of the rate of disappearance of *p*HPP in these reactions showed, however, that the rate of the first oxidation step was strictly comparable in all the flasks and was not proportional to the observed oxygen uptakes. In the same way it was established that the activations were of the *p*HPP step itself.

*The Action of Ascorbic Acid in Enzyme Systems.* The "dissociation constant" of an "ascorbic-acid-enzyme complex" based on the amounts required for tyrosine oxidation is meaningless in view of



the above discussion, and it may still be premature even to call ascorbic acid a coenzyme in this reaction (80). An oxidation-reduction carrier function, for which certain foreign compounds can also substitute, or an indirect effect on some part of this unknown enzyme system, are both equally possible modes of action of ascorbic acid. It is instructive that ascorbic acid has recently been found to act in other types of enzyme systems. Nason et al. have found that a labile oxidized derivative of ascorbic acid participated in the oxidation of DPNH by an enzyme from peas (55). A similar system in yeast has also been described (36). Ascorbic acid was required for the conversion of pteroylglutamic acid to citrovorum factor by a liver enzyme system (56). In the latter system isoascorbic and gluco-ascorbic acids worked equally as well as ascorbic acid, just as they did in the tyrosine system. It would therefore be of considerable interest to know if the other types of compounds found active in the tyrosine system would also act in the formation of citrovorum factor and with the pea and yeast enzymes.

An unusual enzyme, which catalyzed the enol-keto tautomerization of *p*HPP and other phenylpyruvates, was isolated from the tyrosine-oxidizing system (40). Its action was not affected by ascorbic acid, however, and since it has not yet been proved to function in the oxidation of *p*HPP, it will not be further described at the present time.

### HOMOGENTISATE OXIDATION

Our most complete knowledge of tyrosine metabolism has to do with those reactions beyond homogentisate. The already classical study of Ravdin and Crandall (68) established in 1950 the oxidation of homogentisate to the new intermediate, fumaryl-acetoacetate, which they isolated and identified. Its subsequent hydrolysis to fumarate and acetoacetate was done by an enzyme resembling one which had been studied earlier with model substrates (49, 11). Suda and Takeda in Japan found that the homogentisate oxidation reaction involved iron, which could be partially resolved from the enzyme and restored by  $\text{Fe}^{++}$  but not  $\text{Fe}^{+++}$  (82). This iron effect has since



been amply confirmed by Crandall (12), Schepartz (77), and myself and coworkers, and was ingeniously exploited by the Japanese workers to produce a new type of experimental alcaptonuria, by inhibition in vivo of the enzyme in guinea pigs with  $\alpha,\alpha$ -dipyridyl (83).

*Properties of Homogentisate Oxidase as a New Type of Enzyme.* Our own studies of the homogentisate oxidase (37) gave us no indication that more than one step was involved in the reaction; one molecule of  $O_2$  apparently reacted directly. Fractionation and inhibition studies have not dissociated the single reaction into two, benzoquinone acetic acid as a possible intermediate has been generally agreed to be inactive (12, 77, 37), and a coupled peroxidase-oxidase type of reaction like that found in tryptophan metabolism (38) was not concerned. Secondly, the enzyme was inhibited by reagents acting on both ferric and ferrous iron, but it was not inhibited by carbon monoxide. The dissociability of the iron and its failure to react with CO was unlike known porphyrin-iron enzymes reacting with oxygen. A third characteristic of the enzyme was its essential sulfhydryl nature. It was inhibited by the usual types of sulfhydryl reagents, and in the appropriate cases this inhibition could be reversed. The purified enzyme was activated by reducing agents such as glutathione or ascorbic acid, but not by cysteine, which inhibited it, probably by complexing with the iron. This activation was distinct from the activation of the resolved enzyme by reduction of  $Fe^{+++}$  to  $Fe^{++}$  (82), and was merely due to reduction of the essential sulfhydryl groups on the enzyme. The ascorbic acid activation of this reaction should not be used to bolster the erroneous belief in a second specific site of action of ascorbic acid in tyrosine metabolism. This belief, arising from a single report (78) of homogentisate excretion by scorbutic guinea pigs fed excess tyrosine, and confirmed by others only with measurements with the non-specific Briggs reaction (see 60), is continually reasserted in textbooks. Contrary to the impression sometimes given (90, 91), the evidence is quite clear that ascorbic acid acts in tyrosine oxidation specifically at the level of  $p$ HPP oxidation and not later.



The above properties of the homogentisate oxidase—the consumption of one molecule of  $O_2$  in a single step, the participation of iron without a porphyrin in the oxidation, and the presence of essential sulfhydryl groups—would seem to characterize a new type of enzyme. These properties are consistent with the possibility that here is the first example of an iron-sulfhydryl enzyme, employing the same principle as the early iron-sulfhydryl models of respiratory systems studied by Warburg. This possibility, and the nature of some other enzymes which appear to have properties similar to the homogentisate oxidase, will be considered by Crandall. A final point about this enzyme concerns the question of its presence or absence in the liver, or the kidney (13), of the alcaptonuric person. When the opportunity occurs, the point should be tested.

*Maleyl-Acetoacetate, the Immediate Product of Homogentisate Oxidation.* My coworkers and I have used the near-ultraviolet absorption of the products of homogentisate oxidation to identify those products and to follow their formation and removal. In the course of such experiments it was recognized that the product of homogentisate oxidation in our purified system was quite different from the fumaryl-acetoacetate isolated by Ravdin and Crandall. The absorption curves of our product, determined at pH 1 and pH 13 in deproteinized filtrates of the enzyme reaction mixture, can be seen from the solid line in Fig. 9 to have the typical behavior of a  $\beta$ -diketone, with little absorption at pH 1, and at pH 13 a marked absorption referable to its enolate form. Fumaryl-acetoacetate, shown for comparison in the same figure by the dashed curves, has a similar absorption of its enolate at pH 13, but at pH 1 it has its own characteristic absorption which is atypical for  $\beta$ -diketones.

The new product was identified as maleyl-acetoacetate by the similarity of all its functional groups to those of fumaryl-acetoacetate, its reduction to succinyl-acetoacetate, and by its degradation. Both compounds proved to be very similar  $\beta,\delta$ -diketo-dicarboxylic acids, each containing an olefinic group. Alcoholic KOH hydrolysis gave maleic acid from the maleyl-acetoacetate and fumaric acid from the fumaryl-acetoacetate which were identified chromatographically in several solvents by their characteristic  $R_f$ 's.



Two marked differences were noted between these two compounds which differed only in the *cis* and *trans* configurations at their olefinic groups. Maleyl-acetoacetate was not affected by the enzyme which hydrolyzed fumaryl-acetoacetate, a fact used to advantage for the purification of the former compound and in the assay of the isomer-

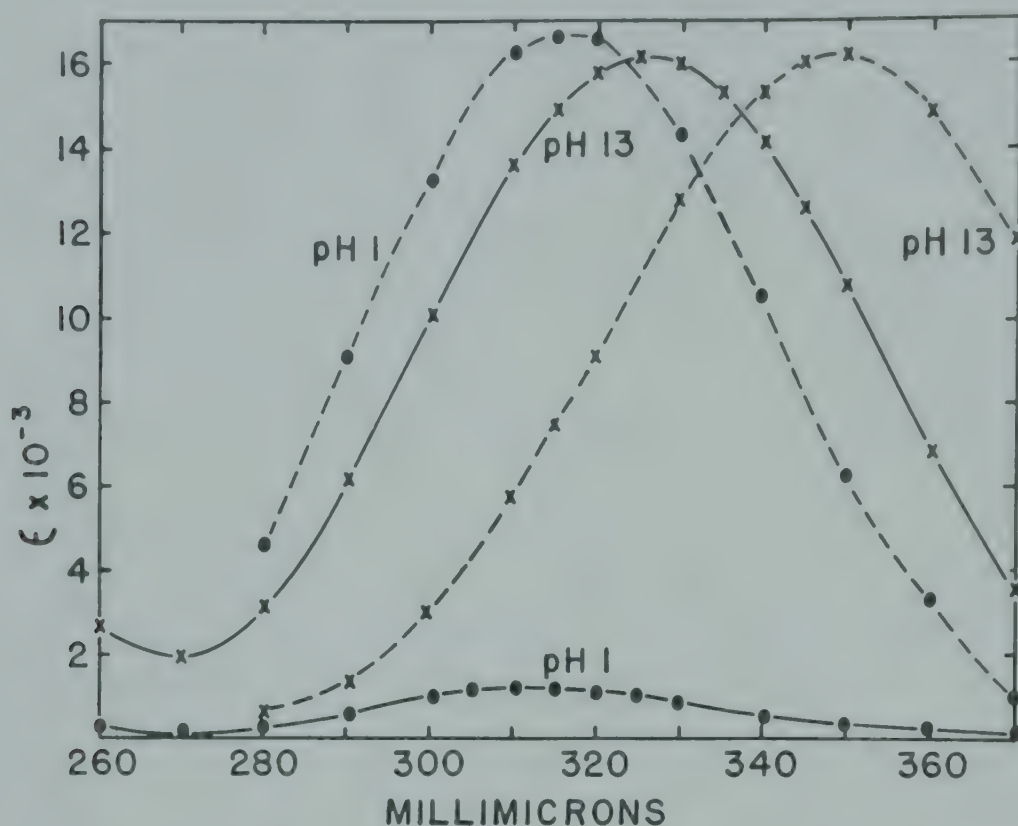


FIG. 9. The absorption curves of maleylacetoacetate (—) and fumarylacetoacetate (----), each at pH 1 (●) and pH 13 (x), (37).

ase reaction which will be described. The other difference was the near-ultraviolet absorptions at pH 1 already mentioned. Since the absorption at pH 13 of both compounds can be attributed to the enolate forms, the very similar absorption of fumaryl-acetoacetate at pH 1 must also be due to its atypical existence as an enol in an aqueous acid solution, maleyl-acetoacetate where is keto. The reason for this stability of the enol of fumaryl-acetoacetate must be sought in its *trans*-configuration. Perhaps this permits a linear all-*trans* enol possessing greater resonance stability than could be attained by maleyl-acetoacetate, where the *cis* configuration would introduce a "hook" in the molecule (Fig. 10), or by other  $\beta$ -diketones in general. The upper left and lower right structures in Fig. 10 represent these compounds as they must exist in acid solutions. Bromine titration of such solutions has demonstrated the rapid uptake of three

molecules of bromine by fumaryl-acetoacetate, a reaction indicating the three unsaturated bonds of the enol form, and on the other hand the uptake of only one molecule of bromine by maleyl-acetoacetate, that one due to the single olefinic group of the keto form.

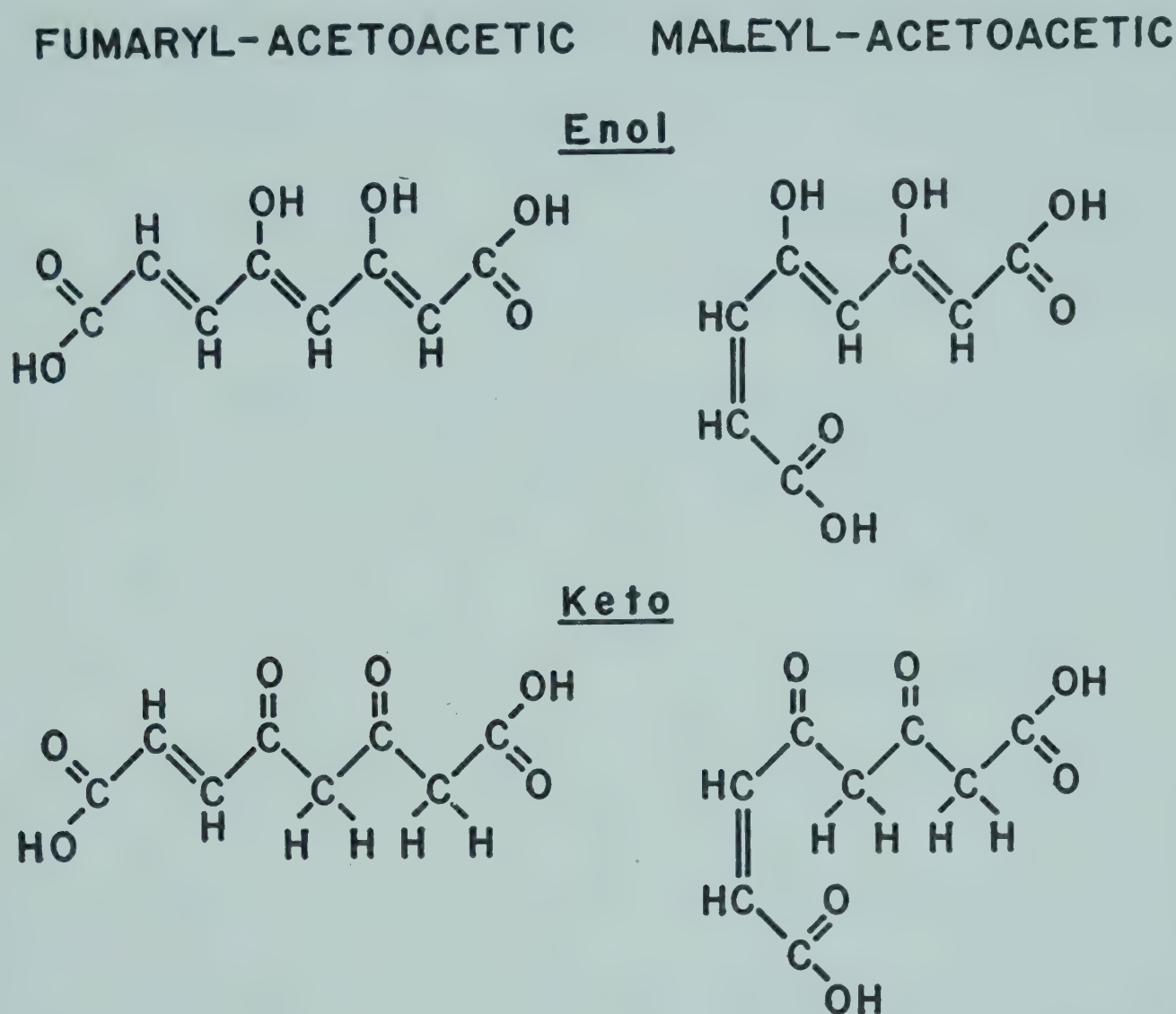


FIG. 10. The enol and keto forms of maleyl- and fumaryl-acetoacetic acids. The absorption spectra and bromine titrations of aqueous acid solutions of each indicated their existence as the enol form of fumaryl-acetoacetic acid (upper left) and the keto form of maleyl-acetoacetic acid (lower right). The extra stability of the all-*trans* enol fumaryl-acetoacetic acid pictured may account for this difference.

*The Enzymic cis-trans Isomerization.* It was a reasonable expectation that a *cis*-compound would be formed upon opening an aromatic ring, and that a maleyl group once formed would necessarily have to be isomerized before its further metabolism would be possible. An enzymic *cis-trans* isomerization had not previously been described, however, although there were indications that such reactions did occur in biological systems (32, 65) or under prophetically mild conditions (52). It was therefore pleasing to be able to isolate the



isomerase of maleyl-acetoacetate, free from the enzyme hydrolyzing fumaryl-acetoacetate, and to demonstrate directly the enzymic conversion of the *cis* to the *trans* by the ultraviolet absorption curves of the compounds before and after the reaction. This reaction could not be followed conveniently, however, and a more useful assay took advantage of the inability of the hydrolyzing enzyme to act on maleyl-acetoacetate. In the presence of excess hydrolyzing enzyme any maleyl-acetoacetate isomerized was immediately split, and the reaction could be followed directly in the spectrophotometer at neutral pH by the disappearance of the absorption of the diketo acid.

*Glutathione as the cis-trans Isomerase Coenzyme.* Fig. 11 shows some assays of the isomerase reaction. Maleyl-acetoacetate and excess hydrolyzing enzyme showed no reaction until isomerase was added. Then (in the uppermost experiment where GSH was already present) isomerization occurred, the fumaryl-acetoacetate formed was split, and the optical density decreased. It can be noted that neither GSH nor isomerase separately caused a reaction. The specific coenzyme function of GSH in this *cis-trans* isomerization was shown by the inability of other sulfhydryl compounds to replace it. The fragments of GSH, cysteinyl-glycine and glutamyl-cysteine, while they did not replace GSH as the coenzyme in the reaction, affected the enzyme so that on subsequent addition of GSH a more rapid reaction ensued. This effect has been traced to the presence on the isomerase itself of essential sulfhydryl groups which are needed for the reaction, in addition to the sulfhydryl group of the glutathione coenzyme.

*The Hydrolyzing Enzyme.* The remarkable specificity of the hydrolyzing enzyme in acting on fumaryl- but not on maleyl-acetoacetate did not at first appear to fit with its known ability to hydrolyze a variety of other diketo acids. [The "acyl-pyruvase" (49) and the "triacetic acid hydrolyzing enzyme" (11) were undoubtedly the same enzyme, and are remembered in the name,  $\beta$ -diketonase, which we have used. Fumaryl-acetoacetate hydrolase, used by Crandall, is physiologically the most appropriate term for this many-named enzyme]. Comparison showed that  $\alpha,\gamma$ -diketovalerate, the



most rapidly hydrolyzed of the other diketo acids (49), was split by the enzyme one-twelfth as rapidly as was fumaryl-acetoacetate. That fumaryl-acetoacetate is probably the natural substrate of this enzyme was also indicated by its much greater affinity (lower  $K_m$ ) for fumaryl-acetoacetate than for  $\alpha,\gamma$ -diketovalerate. A very slow rate

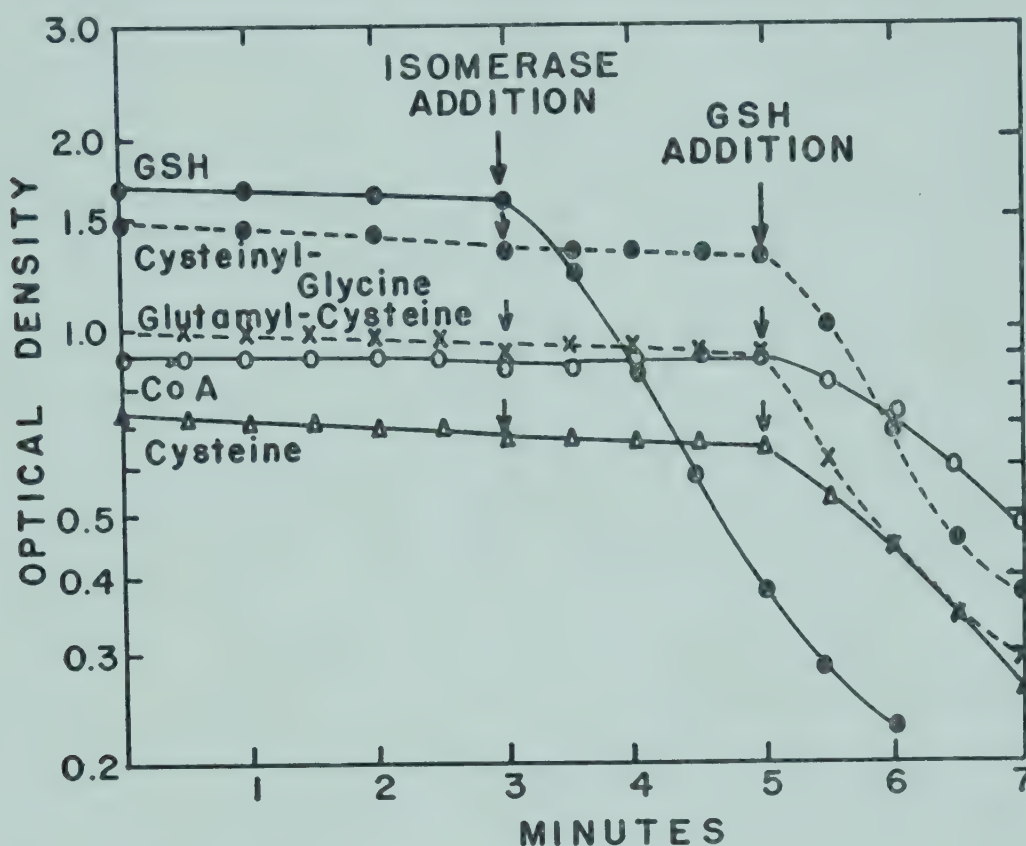


FIG. 11. Assays of the *cis-trans* isomerase spectrophotometrically at 330  $m\mu$ . The absorption of maleyl-acetoacetate in the presence of an excess of the enzyme hydrolyzing fumaryl-acetoacetate falls only when the purified isomerase and glutathione (GSH) are both present. The enzyme or coenzyme separately are ineffective. The specificity of GSH is indicated by the inability of a number of other sulfhydryl compounds to replace it. The reaction approximates first-order kinetics (37).

of hydrolysis of maleyl-acetoacetate, less than 1 per cent of that of fumaryl-acetoacetate, did occur in our purest preparations of the enzyme, but this could not be distinguished from a slow isomerization and subsequent hydrolysis. Such a slow reaction, if actually due to hydrolysis of maleyl-acetoacetate by the enzyme, would be of the same order as the rate found with most of the other diketo acids which have been tested with this enzyme. It would be interesting to determine whether it is the enol form of the other diketo acids which is hydrolyzed, and if it is the amount of a compound existing in the enol form which determines its relative rate of reaction with the enzyme.



Neither the isomerization of maleyl-acetoacetate nor the hydrolysis of fumaryl-acetoacetate appeared to be reversible. Fumaryl-acetoacetate was split by its enzyme without the demonstrable participation of any sulfhydryl groups, and the reaction did not require phosphate nor form products giving the hydroxylamine reaction. The energy of the diketo group was apparently lost in a simple hydrolytic reaction.

### CODA

This history of the metabolism of phenylalanine and tyrosine has indicated the important role of experimentation on the intact animal, probed by genetic, isotopic, and nutritional techniques, in providing a general picture of metabolic pathways. It has also indicated the ultimate need for the more laborious step-by-step demonstration in isolated enzyme systems that the reactions postulated from the intact system *do* occur and that the compounds used *are* intermediates. The criteria by which metabolic intermediates are accepted as such have necessarily been couched in terms of enzyme reactions. These criteria were gradually worked out during the elucidation of the glycolysis pathway, and the most brilliant application of them led to the closure of the tricarboxylic acid cycle. The latter work made these postulates familiar in enzyme chemistry and their use in the present study may be noted: A goes to C through B (the postulated intermediate) *if* it can be demonstrated that A goes to B and B goes to C, each at least as fast as A to C, *and if* a specific alteration of the reaction of A to C alters appropriately either of the reactions A to B or B to C.

On the basis of the above postulates rests our present picture of the reactions of tyrosine metabolism (Fig. 12). The prizes of additional work in this field, as in others, will be the demonstration of the intercalated, unsuspected reactions and intermediates, and the discovery of new enzymes employing new principles to bring about unfamiliar reactions. When this is done, there will be other unexplained phenomena still emanating from the intact-animal experiments, involving even the same reactions, to challenge the enzyme chemist. There is even now the possibility of a alternative route past

homogentisic acid, as indicated by Dakin's experiments, and the selection and control of these alternative metabolic possibilities by ketosis in the alcaptonuric and in the normal person, as indicated by the experiments of Katsch (35) and of Takeda et al. (84).

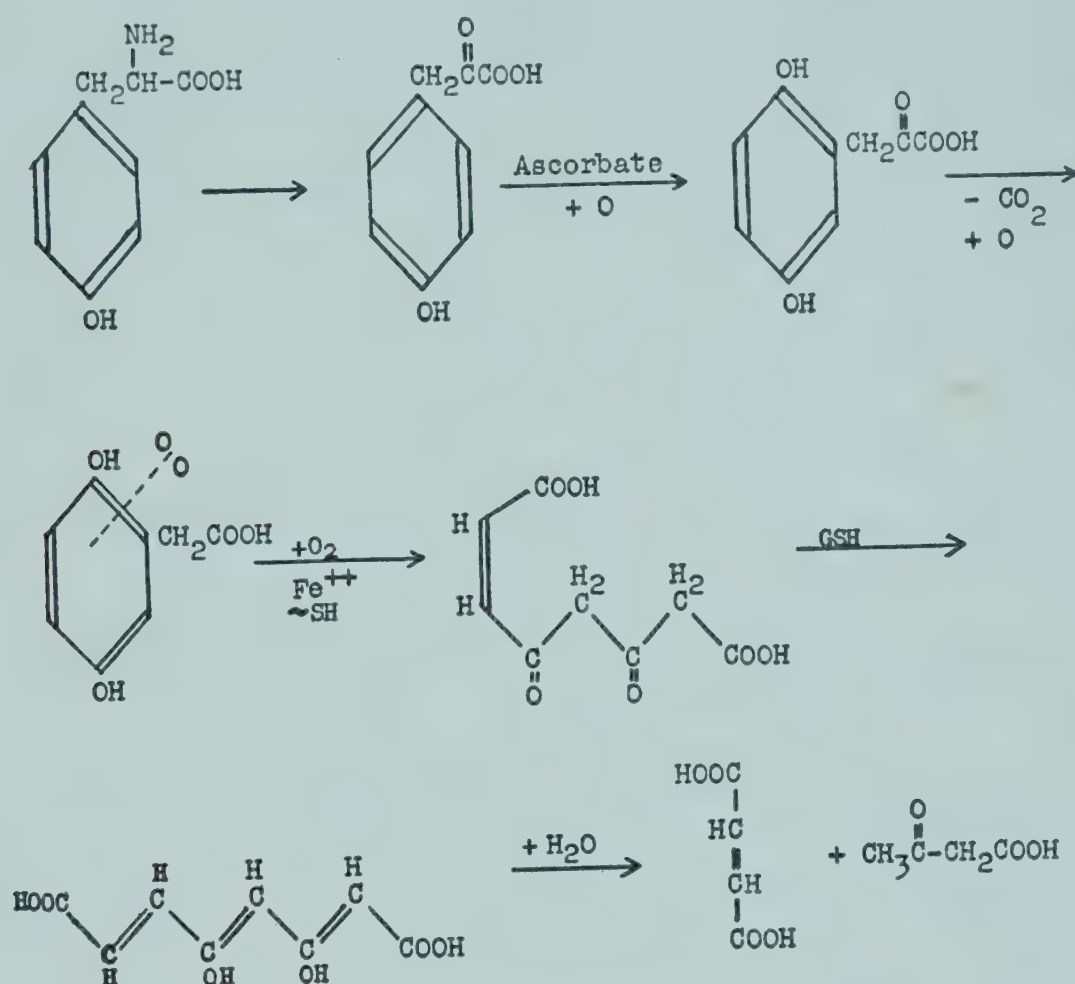


FIG. 12. Diagram of the pathway of reactions of tyrosine metabolism in liver as at present known. The intermediates in the order shown are L-tyrosine, *p*-hydroxyphenyl-pyruvate, 2,5-dihydroxyphenylpyruvate, homogentisate, maleyl-acetoacetate, fumaryl-acetoacetate, and fumarate plus acetoacetate.  $\alpha$ -Ketoglutarate is converted to glutamate with the participation of pyridoxal phosphate in the initial transamination, of which only the conversion of tyrosine to *p*HPP is shown.

Ultimately, to understand the biological functions which have already been observed, we will need to understand not only each of the enzymes in the several pathways, their qualitative presence or absence, as controlled genetically and by disease, but also their quantitative ebb and flow as determined by the physiological state of the individual.



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# THE FERROUS ION ACTIVATION OF HOMOGENTISIC ACID OXIDASE AND OTHER AROMATIC RING-SPLITTING OXIDASES \*

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THE EFFECT of ferrous ions on the liver enzyme system which oxidizes homogentisic acid to fumaric and acetoacetic acids was first noted by Suda and Takeda (1), who observed that the complete loss of activity which follows ammonium sulfate precipitation of the enzyme at pH 5.5 can be partially restored by the addition of ferrous ions, that other metallic ions are ineffective, and that  $\alpha,\alpha$ -dipyridyl inhibits the intact enzyme. Since then, these observations have been confirmed and extended in our laboratory (2), by Schepartz (3), and by Knox (4).

Schepartz has shown that a variety of metal-binding compounds inhibit the enzyme in extracts of rat liver acetone powders, and that losses of activity due to aging, exposure to a low pH, and treatment with versene could be reversed with ferrous ions. We have obtained essentially similar results and have shown that concentrations of ferrous ion as low as  $10^{-5}$  M. will, in the presence of ascorbate, partially reactivate the aged ethanol-precipitated enzyme at pH 7.0. In addition, we have evidence in agreement with Knox (4) that the enzyme contains essential sulfhydryl groups.

## STRUCTURALLY ANALOGOUS ENZYMATIC OXIDATIONS

Three other enzymatic oxidations involving cleavage of the aromatic ring of the substrate in which the overall structural changes are almost identical with those occurring in the enzymatic oxidation

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of homogentisic acid have recently been reported. The enzymatic oxidation product of homogentisic acid has been shown to be 4-

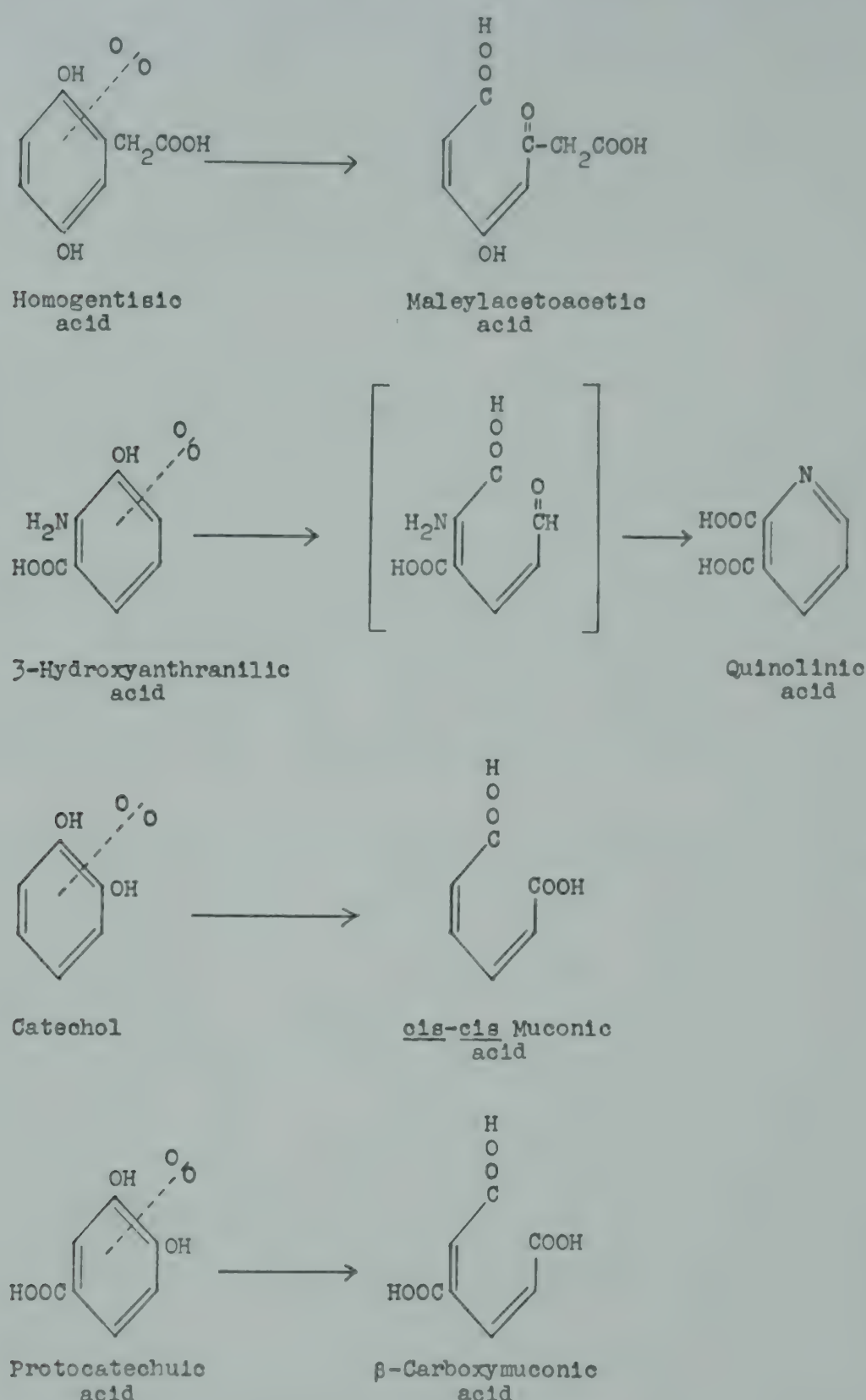


FIG. 1. Structurally analogous oxidations.

maleylacetoacetic acid by Knox and Edwards (5). In the conversion of 3-hydroxyanthranilic acid to quinolinic acid in the rat, the amino nitrogen of 3-hydroxyanthranilic acid is incorporated into the pyridine ring of quinolinic acid by a process involving ring rupture

between carbons 3 and 4, followed by ring closure, according to evidence obtained by Schayer and Henderson with tryptophan labeled with  $N^{15}$  in the indole ring (6). The detection of intermediates when this reaction has been conducted with liver enzyme systems has been reported by Schweigert et al. (7, 8, 9) and by Henderson (10), who demonstrated that two atoms of oxygen are used in this conversion. The resulting oxidation product which undergoes ring closure to form quinolinic acid is presumably the dicarboxylic amino aldehyde shown in brackets in Fig. 1 (9). Hayaishi and Hashimoto (11) and Evans and Smith (12) have evidence that *cis-cis* muconic acid is the immediate product of catechol oxidation by *Pseudomonas*. MacDonald, Stanier, and Ingraham (13) have demonstrated that  $\beta$ -carboxymuconic acid is the product of protocatechuic acid oxidation by *Pseudomonas fluorescens*. These oxidations are depicted in Fig. 1. In each case it appears that, in effect, two oxygen atoms have been added across an aromatic carbon-to-carbon bond, either adjacent to a phenolic group or between two phenolic carbons. The resulting rupture of this bond is therefore accompanied by the oxidation of phenolic carbons to carboxyl groups and of nonphenolic carbons to carbonyl groups.

### COMPARISON OF THE ENZYMES

It is not surprising that the two enzymes catalyzing the oxidation of 3-hydroxyanthranilic acid and catechol, respectively, are similar to homogentisic acid oxidase in being reactivated by ferrous ions under certain conditions. Miyake, Bokman, and Schweigert (8, 9), and Henderson (10) have reported the inhibition of 3-hydroxyanthranilic acid oxidation in homogenates and acetone powders of rat liver by several metal-binding inhibitors, and the reversal of this effect by ferrous ions. Suda et al. (14) and Sistrom and Stanier (15) have evidence that ferrous ion activates the enzymatic cleavage of catechol. These effects are not exhibited, however, by a ten-fold purified preparation of protocatechuic acid oxidase prepared by Stanier and Ingraham (16). These results are summarized in Table 1.



TABLE 1

COMPARISON OF FERROUS ION-ACTIVATION OF RING-SPLITTING ENZYMES

Enzyme	Inhibition	Reversal of Inhibition	Reference
Homogentisic Acid Oxidase	Low pH	Fe <sup>++</sup> + ascorbate	1, 2, 3, 4
	H <sub>2</sub> S Cysteine		
	KCN Bipyridyl		
	Versene	Fe <sup>++</sup>	
	Oxine		
	<i>o</i> -Phenanthroline		
	<i>p</i> -Chloromercuribenzoate (Activation by thiols)	Fe <sup>++</sup> + GSH	
-----			
3-Hydroxy-anthranilic Acid Oxidase	Bipyridyl	Fe <sup>++</sup>	8, 9, 10
	Oxine	Fe <sup>++</sup>	
	Versene	Fe <sup>++</sup>	
	Citric acid	Fe <sup>++</sup>	
-----			
Pyrocatechase	Dialysis (Activation by Fe <sup>++</sup> and by glutathione)	Fe <sup>++</sup>	14, 15
-----			
Protocatechuic Acid Oxidase	<i>p</i> -Chloromercuribenzoate (Unaffected by versene)	GSH	16

It should be noted that a ferrous ion requirement was not shown even by crude protocatechuic acid oxidase preparations (16), and that in similar studies of pyrocatechase which are under way Sistrom and Stanier have found that ferrous ion requirements are intensified by purification (15). This suggests that the ferrous ion activation observed in crude preparations of homogentisic and of 3-hydroxy-anthranilic acid oxidase may not be artifacts which will be eliminated by further purification.

The sharp contrast between pyrocatechase and protocatechuic acid oxidase awaits further explanation. In my own opinion this striking difference may be quantitative rather than qualitative and does not preclude the possibility that iron is a component of all these enzymes but is more firmly bound in the case of protocatechuic acid oxidase. A quantitative difference between pyrocatechase and homogentisic acid oxidase is indicated by the report that the former is highly resistant to inhibition by bipyridyl (14).

## FURTHER PROPERTIES OF HOMOGENTISIC ACID OXIDASE

My coworkers and I have been particularly interested in the effect of pH on the activity of homogentisic acid oxidase in the presence

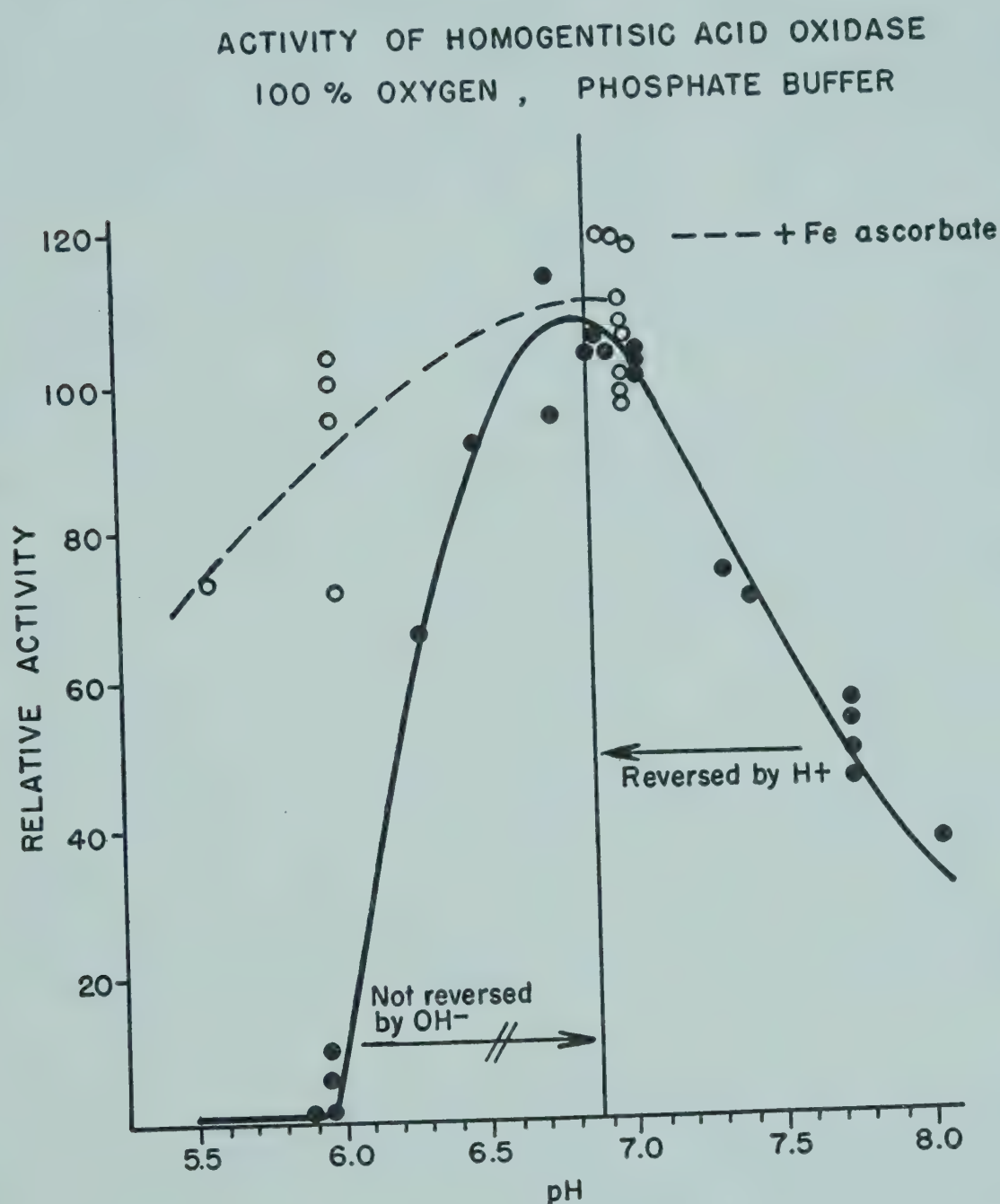


FIG. 2. pH activity curve. Solid dots, enzyme and substrate alone. Open circles, enzyme and substrate supplemented with  $10^{-3}$  M.  $\text{Fe}^{++}$  and  $6 \times 10^{-3}$  M. ascorbate.

and absence of ferrous ions in combination with ascorbate and with reduced glutathione (GSH). The pH-activity curve is shown in Fig. 2. The crude enzyme preparation was obtained from homogenized rat liver by centrifuging at  $100,000 \times g$  and discarding the precipitate.

The curve shows that in phosphate buffer the pH optimum is



approximately 7.0 and that the inhibition obtained by raising the pH above the optimum is readily reversed by returning the pH to 7.0. On the acid side of the pH optimum, the activity is completely lost at pH 6.0 and is not restored by raising the pH to 7.0. With ferrous ascorbate, however, this inhibition is largely reversed at pH 6.0 and partially reversed at pH 5.5. Under these conditions (pH 6.0) ascorbate alone is ineffective, and ferrous ion alone or a combination of ferrous ion and GSH is markedly inferior to ferrous ascorbate. The results suggest that the loss of activity at pH 6.0 may be due to a dissociation from the enzyme of ferrous ion, which then must become unavailable for recombination with the enzyme when the pH is raised to 7.0 because of interfering substances present in the crude enzyme preparations. If the intact enzyme is an iron-protein, the bond between the iron and the enzyme is stable above pH 7.0 (not broken by dialysis) and is labile at pH 6.0.

We have also observed that ferrous ions are required in addition to GSH to reactivate the enzyme after treatment with *p*-chloromercuribenzoate (PCMB). Activation of untreated preparations of the enzyme by GSH and thiols other than cysteine has been observed (2, 3, 4); and although relatively insensitive to iodoacetate and maleate, our crude enzyme preparations are strongly inhibited by iodosobenzoate and mercuric chloride in addition to PCMB. The activity therefore appears to depend upon reduced sulfhydryl groups.

TABLE 2

## HOMOGENTISIC ACID OXIDASE

Reversal of Inhibition by PCMB at pH 7.0

Incubation	Activity $\mu\text{l. O}_2/\text{min.}$
Enzyme	18.0
Enzyme + PCMB	1.9
Enzyme + PCMB + GSH	2.2
Enzyme + PCMB + $\text{Fe}^{++}$	2.0
Enzyme + PCMB + $\text{Fe}^{++}$ + GSH	18.0
Enzyme + $\text{Fe}^{++}$ + GSH	19.0

PCMB  $10^{-3}$  M.; GSH  $10^{-2}$  M.;  $\text{Fe}^{++}$   $10^{-3}$  M.

It may be seen in Table 2 that the enzyme is almost completely inhibited by  $10^{-3}$  M. PCMB and that the loss of activity is completely unaffected by the subsequent addition of either GSH or  $\text{Fe}^{++}$ . In the presence of a combination of GSH and  $\text{Fe}^{++}$ , however, the activity is completely restored. This observation suggests that possibly iron is linked to the enzyme through its sulfhydryl groups, as proposed by Knox (17), and that a working hypothesis for the inhibition of the enzyme by PCMB and its reversal by  $\text{Fe}^{++} + \text{GSH}$  may be represented by the following equations:



The losses of activity which occur on the acid side of the pH optimum are consistent with equation 3. Further investigation of these effects in purer preparations will be necessary to establish their validity and significance.

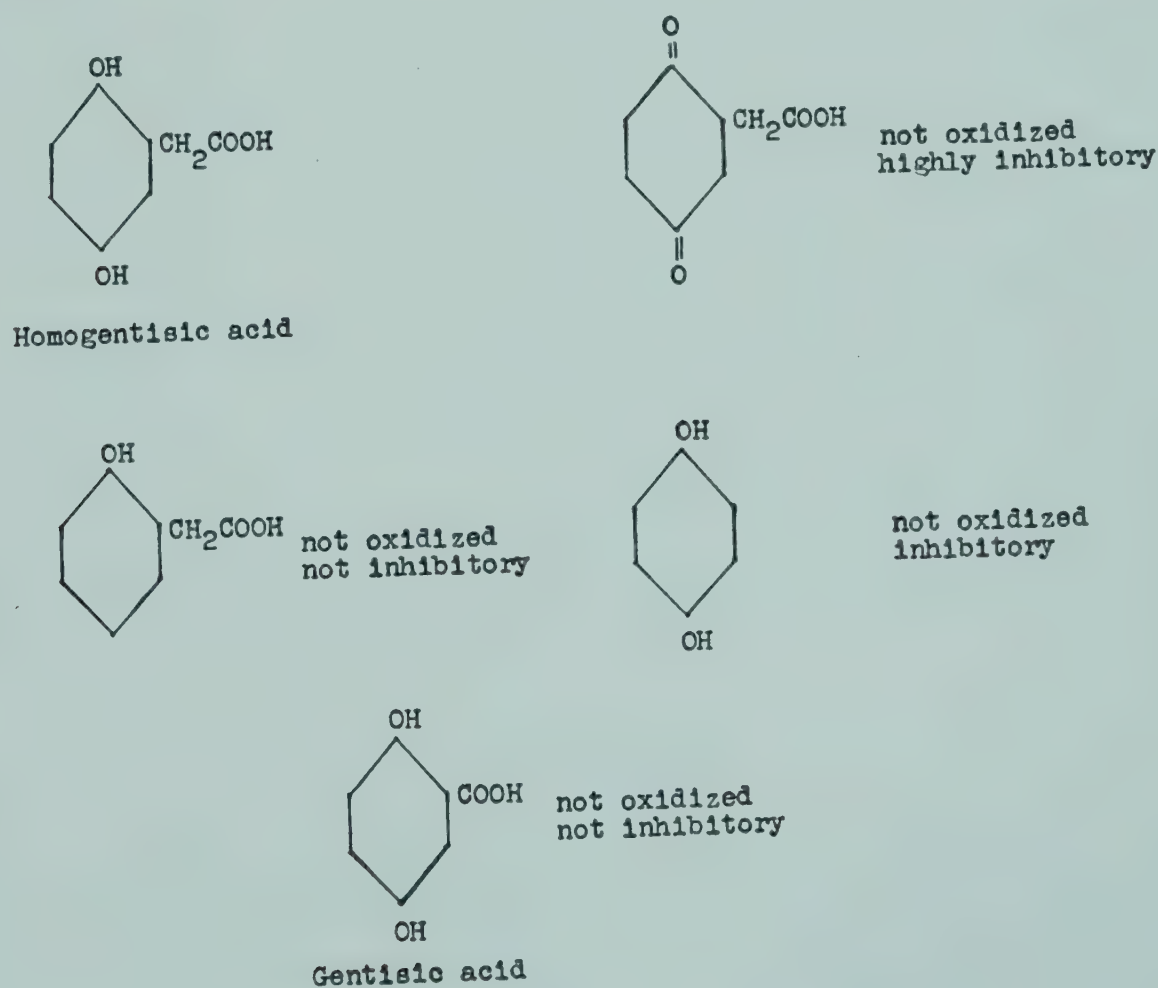


FIG. 3. Specificity of homogentisic acid oxidase.



We have found our preparations to be highly specific for homogentisic acid oxidation since, as shown in Fig. 3, neither *o*-hydroxyphenylacetic or gentisic acids were oxidized. The inhibitory action of hydroquinone has been reported by Schepartz (3). In agreement with Schepartz (3), we find that benzoquinone acetic acid, a postulated intermediate in this oxidation, is not oxidized by this system and, in fact, is highly inhibitory. The possibility remains, however, that it may nevertheless be a highly transient intermediate, and non-inhibitory in the infinitesimally small concentrations which might exist during the course of the oxidation.

### CONCLUSIONS

It should be emphasized that much of the evidence that ferrous ions specifically activate these aromatic ring-splitting oxidases has been obtained with crude enzyme preparations and that the possibility exists that highly purified preparations will not exhibit these effects. Suzuki et al. (18) claim in a preliminary report to have a preparation of homogentisic acid oxidase which is activated most effectively by a combination of vitamin B<sub>12</sub>, folic acid, adenylic acid, ascorbic acid, glutathione, and DPN, and which does not respond to ferrous ions. On the other hand, Knox has observed ferrous ion activation after 50-fold purification (4). Although the study of these enzymes is still in a preliminary stage and definitive results with highly purified preparations have not yet been obtained, the available evidence suggests that they are members of a new class of metallo-protein enzymes which catalyze the oxidative rupture of the aromatic rings of certain phenolic compounds at the bond adjacent to the phenolic group.

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# CONVERSION OF PHENYLALANINE TO TYROSINE

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ALTHOUGH IT HAS been known for a long time that animals can form tyrosine from phenylalanine, this conversion was only recently demonstrated at the enzymatic level (7). This type of reaction, conversion of an aromatic compound to a phenol, is a common occurrence in animals. Thus tryptophan is converted to 5-hydroxytryptophan, kynurenine to 3-hydroxykynurenine, anthranilic acid to 3-hydroxyanthranilic acid. Foreign compounds such as aniline, acetanilide, diphenyl, quinoline, and many others are also readily converted to phenols in all animals.

It is unlikely that this type of reaction is reversible. Certainly the enzyme responsible for converting phenylalanine to tyrosine does not catalyze the reverse reaction. Experiments with C<sup>14</sup>-labelled tyrosine in rats, dogs, and rabbits indicate that conversion to phenylalanine does not take place in vivo to any detectable extent (Table 1), contrary to the findings of Ågvist (1).

TABLE 1

CONVERSION OF C<sup>14</sup>-PHENYLALANINE TO TYROSINE IN VIVO

Animal	Dose	Serum Albumin Phenylalanine	Serum Albumin Tyrosine
		c.p.m./ $\mu$ M.	c.p.m./ $\mu$ M.
Dog 1	20 $\mu$ c. 3-C <sup>14</sup> -DL Phenylalanine	16,900	6,800
Dog 2	20 $\mu$ c. 2-C <sup>14</sup> -DL Tyrosine	0 $\pm$ 5	13,700
Rat	0.5 $\mu$ c. 2-C <sup>14</sup> -DL Tyrosine	0 $\pm$ 7	41,000

Experiments in this laboratory indicate that the enzyme system responsible for the conversion of phenylalanine to tyrosine is distinct from the other hydroxylating enzymes and is specific for L-phenylalanine. In animals it is found only in the liver, from which it can be obtained in a soluble form completely free from microsomes, mitochondria, nuclei, etc.

The studies carried out on the animal phenylalanine-hydroxylating enzyme have been fairly successful, in that enzymes have been purified and cofactors have been found (5). However, as the studies have progressed, it has become more and more evident that enzymatic conversion of phenylalanine to tyrosine is a complex process. The requirements which are now known are the following: Two distinct protein fractions obtained by ammonium sulfate fractionation of liver (Enzyme I and Enzyme II), DPN, aldehyde, oxygen, and  $\text{Fe}^{++}$ . One of the protein fractions can be found in kidney as well. DPN and DPNH can be used interchangeably, although DPNH appears to be more active at low concentrations. Except for formaldehyde, most simple aldehydes are active. Benzaldehyde has been used in most of the studies because of its low volatility and ease of handling. It has not been possible to substitute any dye for the oxygen. The evidence for  $\text{Fe}^{++}$  is based on the almost complete inhibition produced by  $\alpha$ - $\alpha$ -bipyridyl and the fact that preparations inactivated by dialysis against bipyridyl can be reactivated by  $10^{-4}$  M.  $\text{Fe}^{++}$ .

The over-all reaction can be broken down into two steps. First, Enzyme II is preincubated with DPN, and the reaction mixture is heated at  $55^{\circ}\text{C}$ . for 5 minutes to destroy enzymatic activity. Then this heated incubation mixture is added to a flask containing Enzyme I, benzaldehyde, and L-phenylalanine and incubated aerobically to produce tyrosine. Further experiments have indicated that DPN stabilizes Enzyme II and suggest their relationship. The role of benzaldehyde in this reaction and its fate are as yet unanswered. This is due mainly to analytical difficulties in assaying this compound in quantities less than 1 micromole. Experiments with  $\text{C}^{14}$ -benzaldehyde are planned. Alcohol dehydrogenase is not involved, since benzaldehyde is not a substrate of this enzyme. Benzoic acid and



perbenzoic acid cannot replace benzaldehyde, but benzyl alcohol does possess activity. Experiments by Strehler (6) indicate the involvement of DPN and fatty aldehydes in bioluminescence of cell-free extracts of *Achromobacter fischeri*. He suggests a general function of fatty aldehydes in biological oxidations and peroxidations.

Conversion of L-phenylalanine to tyrosine can also take place in certain microorganisms. *B. salmonicida* and phenylalanine-adapted *Pseudomonas*, when incubated with phenylalanine, produce sufficient tyrosine to permit detection in the medium. The conversion of phenylalanine to tyrosine by *Vibrio* O1 was recently demonstrated by Dagley et al. (4). Thus far it has not been possible to obtain active cell-free extracts of a bacterial phenylalanine hydroxylase. In fact, no requirement of the animal's system can be replaced by cell-free bacterial extracts.

It has recently been possible partially to purify the enzyme system in liver responsible for converting aniline to *p*-aminophenol and acetanilide to *p*-hydroxyacetanilide. There is no question that this is a system distinct from the L-phenylalanine-hydroxylating enzyme. A comparison of some of the properties of the two enzymes is presented in Table 2.

TABLE 2

COMPARISON OF L-PHENYLALANINE AND ANILINE HYDROXYLASES FROM LIVER

Property	Phenylalanine Enzyme	Aniline Enzyme
Coenzyme	DPN	TPN
Localization	Soluble	Microsomes
Destruction overnight 3-5° C. (crude)	0-10%	100%
Ketoacid inhibition $10^{-3}$ M.	+	0
2,4-dinitrophenol	0	+

Although there is no evidence to indicate that the specific L-phenylalanine-hydroxylating enzyme forms other than *p*-hydroxyphenylalanine, recent findings indicate that phenylalanine can be hydroxylated in vivo in the ortho and meta positions as well. These

conclusions are based on the findings of ortho and meta hydroxyphenylacetic acid in urine (3, 2). In our laboratory we have administered  $C^{14}$ -phenylalanine to phenylketonuric persons, who excrete unusually large amounts of *o*-hydroxyphenylacetic acid. The isolated acid was found to be highly labelled, an observation indicating its derivation from phenylalanine. Ortho- and metatyrosine may be intermediates in these conversions.

The mechanism for hydroxylating phenylalanine is not at all apparent. Thus far, hydrogen peroxide has not been implicated. The addition of D-alanine and D-amino acid oxidase does not stimulate the oxidation of phenylalanine, nor does catalase inhibit it. The marked inhibition by pyruvate would usually suggest  $H_2O_2$ . However, this inhibition is not overcome by generated  $H_2O_2$ . The involvement of DPN and aldehyde suggests a completely different type of oxidation. Exactly how the benzene ring is attacked is still obscure.

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## DISCUSSION

DR. COHEN: I think the previous speakers in this amino acid symposium have been indifferent to the fate of the amino group in this series of amino acids. I just wanted to mention a tyrosine transaminase which is being studied in our laboratory by Mrs. Canellakis. It has been purified approximately 100-fold from dog liver. It appears homogeneous in the ultracentrifuge but can be resolved into several components electrophoretically. The activity is associated with an intensely blue protein. This protein has been shown to contain copper. Whether or not the blue-protein is necessarily a part of the transaminase is still open to question, but I think that perhaps teleologically it might be of some interest to point out that this copper-protein might be tied up in the oxidation of these aromatic substances as part of a larger



protein complex. This transaminase is remarkably specific in the sense that it reacts with only alpha-ketoglutaric acid, and the specificity extends to the para position of the tyrosine. That is, a variety of compounds substituted in the para position are either competitive inhibitors or do not react at all. I think it would be of some interest to clarify finally the transamination step and its possible relationship to the over-all metabolism of tyrosine.

DR. KNOX: I am very pleased to hear about this transaminase. That is something I was hoping Dr. Cohen would do. I am also quite interested that he finds it specific for alpha ketoglutarate, since this brings up a rather interesting point in the earlier history of tyrosine metabolism—that is, they did not find transamination, because they looked for glutamic acid and this did not accumulate. Later alanine was found and isolated in stoichiometric amounts from the reaction. When we finally did find glutamic acid accumulation in our system we suggested that the alanine in the cruder system had been formed by a second transamination—that is, from tyrosine to glutamic, and from glutamic to alanine. Felix has indicated that the activation with pyruvate we both observed might be due to its direct participation in the transamination. Do you find any evidence for a pyruvic transaminase?

DR. COHEN: Not in the purified system. I would suspect that pyruvic acid would be active with the crude system, but with the highly purified enzyme it is completely inactive.

DR. CANTONI: Did I understand Dr. Crandall to say that the reactivation of the enzyme at pH 6 is with iron and ascorbic acid, or was the acidification carried out in the presence of iron and ascorbic acid?

DR. CRANDALL: The enzyme which has been previously inactivated by acidifying to pH 6.0 was then reactivated by iron and ascorbic acid.

DR. UDENFRIEND: What name shall we give enzymes which hydroxylate the aromatic ring?

DR. STANIER: I think hydroxylase is a good name. I would like to say that for many years we have been trying to demonstrate hydroxylation steps in bacterial systems, but so far we have had a total lack of success in finding extracts with sufficient activity to test.

DR. L. MILLER: We have heard earlier in this session about nonenzymatic iron-catalyzed oxidations. I have heard Dr. Brody describe to some extent very active oxidation of aromatic rings catalyzed by iron and ascorbic acid. I wonder whether either of these factors has been examined in connection with enzymatic oxidations.

DR. UDENFRIEND: Our work on the iron and ascorbic acid-versene model system does not seem to be easily interpreted. We discovered, as Dr. Stadtman did, that we had a wonderful enzyme system that could be boiled and still react very nicely. It turned out to be iron, ascorbic acid, and versene.

We did discover that the patterns of hydroxylation with this system were similar to the type that occur in vivo. The results were not comparable with those obtained with hydrogen peroxide, and we showed that this model system does not act by virtue of hydrogen peroxide production, as one might suspect, but by some active intermediate of ascorbic acid. I suspect that the intermediate may be analogous to that suggested by Nason and by Racker. We have also shown that in vivo ascorbic acid is involved in the hydroxylation of compounds other than phenylalanine. Oxidation of aniline, acetanilide and antipyrine are diminished in scorbutic guinea pigs. Supplementing the diet with ascorbic acid restores their metabolism to normal. It may be that ascorbic acid is generally involved in aromatic hydroxylation.

DR. McELROY: I would just like to ask if you have tried organic peroxides, since aldehyde in the presence of iron will give rise to these compounds and this could lead to a peroxidation.

DR. UDENFRIEND: We did. Dr. Bernard Witkop made up several but these were inactive. We found that requirement for this model system was an enediol or its diketo analogues. Above pH 7 only the enediol compounds were active in our system.

DR. NASON: I was wondering with respect to Dr. Crandall's observation on the reversal of the parachloromercuribenzoate inhibition, which requires both glutathione and iron, is it possible that high concentrations of glutathione inhibit the oxidizing system by binding the iron and that when you reverse parachloromercuribenzoate inhibition by glutathione traces of glutathione are being used to bind some of the iron and it is therefore necessary to add iron to that system.

DR. CRANDALL: We found that the concentration of glutathione used had no effect on the intact enzyme.



# METABOLISM OF TRYPTOPHAN

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THE SUBJECT of tryptophan metabolism includes a large variety of reactions. The mass of this accumulation of generally unfamiliar structures has prompted one of my colleagues to comment that a more appropriate name than *tryptophan*, a compound which appears in tryptic digests, would be *kryptophan*, a compound which appears more obscure as more information accumulates. Actually, the interrelations between various compounds have been fairly clearly established during the last several years. Many disciplines have drawn together to contribute to our knowledge of tryptophan metabolism. Progress in this field has been stimulated by work in various sciences, and these, in turn, have been advanced by the information gained in the study of tryptophan.

## SYNTHESIS OF TRYPTOPHAN

Tryptophan is unique among amino acids in that it contains an indole nucleus. The detailed mechanism of tryptophan synthesis has been only partially revealed. In the foggy picture, the final step has emerged in the case of certain microorganisms. Early studies by Fildes (24) and by Snell (81) indicated that tryptophan could be formed from indole. It has been shown clearly by Umbreit, Wood, and Gunsalus (98), and confirmed by Yanofsky (110), that a condensation between indole and serine (Fig. 1) is catalyzed by a pyridoxal-phosphate-requiring enzyme. This reaction is apparently closely related to, but distinct from, the tryptophanase reaction (75), which has been studied by many investigators whose work was recently reviewed by Happold (30). Wood, Gunsalus, and Umbreit

also showed that tryptophanase is another pyridoxal-phosphate enzyme which splits tryptophan into indole, pyruvate, and ammonia (109). These reactions represent the less familiar types of substitution and activation considered by Metzler, Ikawa, and Snell (68) in their interesting speculations on the mechanism of pyridoxal-catalyzed reactions. These reactions offer a potential site for investigating the factors of enzyme specificity; that is, the reason why in one case serine, and in the other pyruvate and ammonia, are involved. A study of the thermodynamics of these reactions should

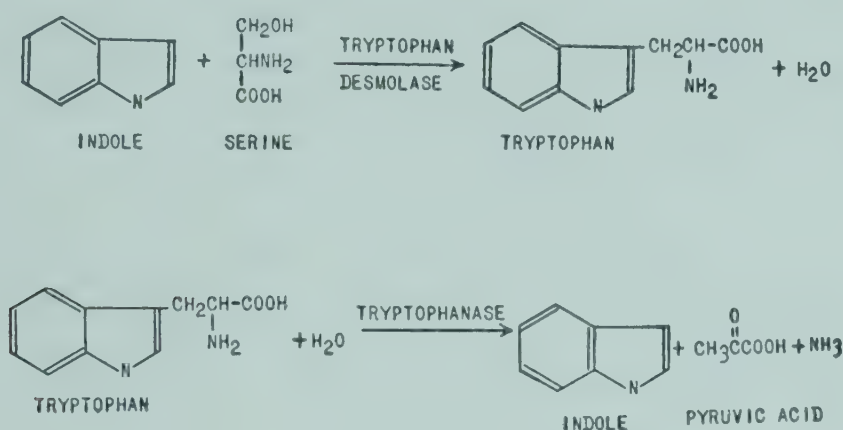


FIG. 1.

also prove of great interest, since it has been shown that serine is dehydrated and deaminated to give pyruvate and ammonia (15). It will be convenient to have a measure of the free energy of reaction in the case of tryptophan synthesis and breakdown, since the sum of these equals the serine to pyruvate plus ammonia reaction. If the tryptophan reactions are indeed irreversible, as has been hinted by previously mentioned workers, the free energy of the serine degradation must be extremely large. Additional information on the nature of the tryptophanase reaction as revealed by effects of substitution of the indole nucleus, has been reviewed by Happold (30). Beerstecher and Edmonds (3) have reported that both indole and pyruvic acid stimulate tryptophanase, but since intact cells were used, the significance of these results is not certain.

There is no problem after yesterday's meeting in obtaining serine for the condensation reaction, but indole is another matter (Fig. 2). Anthranilic acid was found by Snell (81) to replace indole in satisfying the tryptophan requirements of some bacteria. The genetic studies of Tatum, Bonner, and Beadle (90) indicate that anthranilic



acid (a product of tryptophan degradation) is involved in a cycle leading back to indole. Similar genetic evidence implicates nicotinic acid in tryptophan synthesis, since some mutants of *Neurospora* can grow on media supplemented with either the amino acid or the vitamin (2); thus each compound is implicated in the synthesis of the other. But whereas tryptophan is a precursor of the elements of nicotinic acid, as we shall discuss soon, the catalytic role of nicotinic acid in tryptophan synthesis remains unclear.

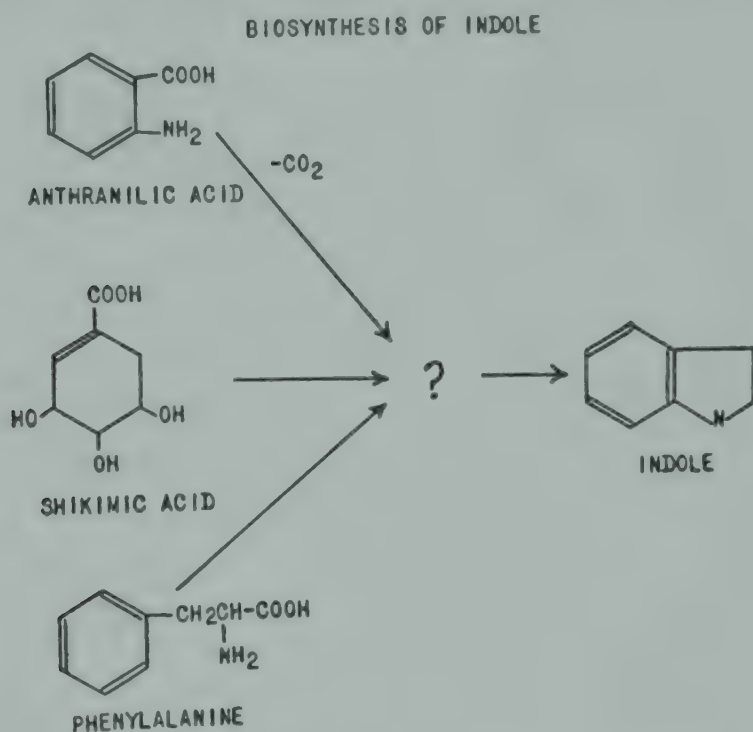


FIG. 2.

Another structure glimpsed through the fog surrounding the mechanism of tryptophan synthesis is found in the studies on aromatization presented in this symposium by Davis (20). The multiple mutants of *Escherichia coli* whose aromatic requirements are met by shikimic acid have a tryptophan requirement in the absence of this versatile precursor (or its progenitors). Since the studies of Davis indicate that *E. coli* uses indole for tryptophan synthesis, some mechanism for the conversion of shikimic acid to indole probably occurs. This may or may not proceed via anthranilic acid, since it has been shown by Nyc et al. that the carboxyl group of anthranilic acid does not persist in the indole ring (76). The fact that phenylalanine can serve as a precursor of indole in the experiments of Haskins and Mitchell with *Neurospora* (31), but not in the multiple-mutant *E. coli* of Davis permits the consideration

of a variety of mechanisms by which indole might be formed. It is obvious that a fruitful field for investigation with genetic and enzyme methods remains in this area.

### DEGRADATION OF TRYPTOPHAN

One pathway for tryptophan destruction has already been mentioned, the hydrolysis to indole, pyruvate, and ammonia. Three other pathways are also known. These are all of particular interest because they lead to the formation of other compounds of great biological significance. One pathway involves hydroxylation of the ring and leads ultimately to the formation of serotonin and related pressor amines. Another reaction sequence retains the indole ring and modifies the sidechain to form indoleacetic acid, an auxin. This sequence and the subsequent reactions of the plant hormone are of undeniable significance in plant physiology. A third mechanism of tryptophan degradation involves oxidative cleavage of the indole ring. This last pathway is a common beginning from which several routes diverge. The path of greatest general biological interest is that leading to nicotinic acid formation. A branch of this route is the formation of quinoline derivatives, kynurenic and xanthurenic acids. Another divergence, which has been of sufficient interest in itself to occupy an enthusiastic group of geneticists and chemists, is the formation of pigments in insects. This route has led to some of the fundamental steps in our knowledge of the oxidative pathway. Finally, the oxidation of tryptophan may be continued to supply the carbon and energy requirement of some organisms.

### 5-HYDROXYINDOLE COMPOUNDS

The enzymatic steps in the formation of compounds of the 5-hydroxyindole series have been explored by Udenfriend and his collaborators. These studies, to be presented later, have been of great value in determining the mechanism of synthesis of the pharmacologically potent substances serotonin and the related bufotenin, and the biological fates of these compounds (Fig. 3).



Early studies of Werle (101) showed the formation of a pharmacologically active substance from tryptophan by kidney preparations. This was assumed to be tryptamine, and the decarboxylation of tryptophan is generally listed in textbooks. This reaction is now considered not to exist. Instead, decarboxylation occurs only after hydroxylation to yield 5-hydroxytryptophan (96). Isotope trapping demonstrated the biosynthesis of this compound, which is rapidly decarboxylated to form 5-hydroxytryptamine (97), recently identified

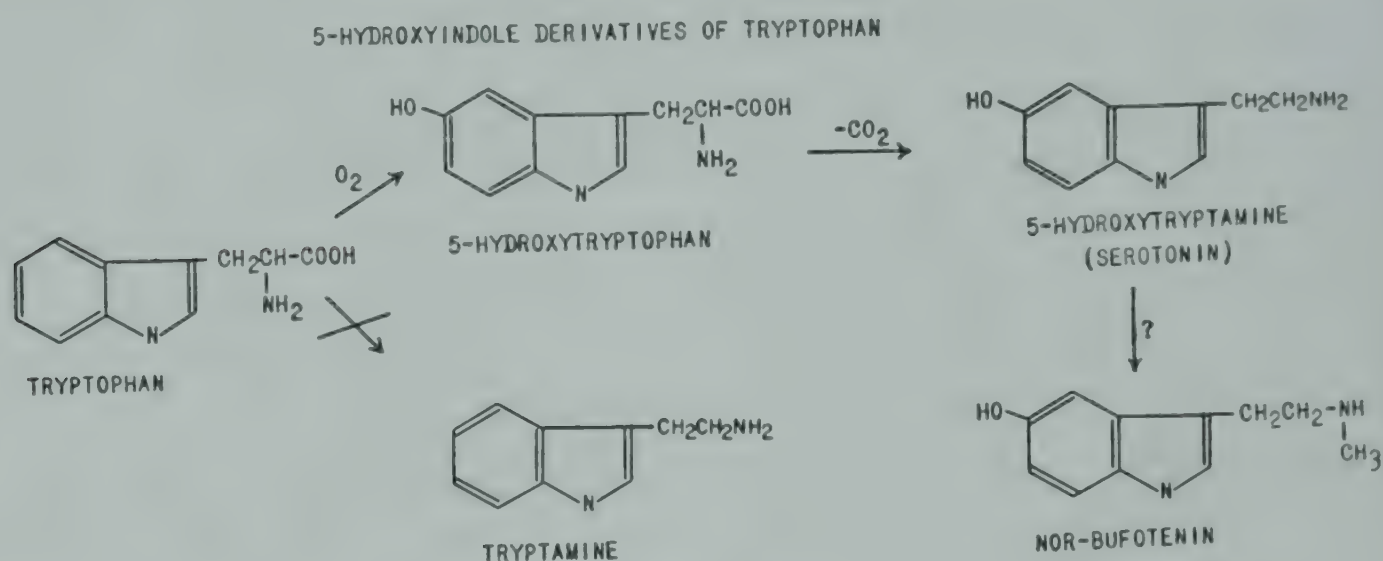


FIG. 3.

with serotonin. The finding that both free and methylated 5-hydroxytryptamine exist in toad venom (103) was interpreted to imply that methylation follows decarboxylation (95). The metabolism of N-methyl tryptophan (abrine) has been studied in many laboratories (27, 28, 59, 60, 61, 88). The results indicate that the primary reaction of this compound in kidney is oxidative demethylation, and not the formation of the corresponding hydroxylated tryptophan. Recently Udenfriend and Titus have found indications for 5-hydroxyindole derivatives with other side-chains, and I suspect that we shall hear more immediately hereafter about these compounds in an evaluation of the role of 5-hydroxytryptophan in animal metabolism. That it is of general importance is evident from the findings of Erspamer (12), which show that the invertebrate hormone, enteramine, is identical with serotonin studied by Rapport et al. (78) and others (22).

## INDOLE-CONTAINING AUXINS

The nature and mode of action of plant auxins are interesting subjects beyond the scope of this review. The identification of certain indole derivatives in plants was made in the process of identifying these stimulating compounds.

The relevance of auxin studies to tryptophan metabolism (Fig. 4) was established by Thimann when he demonstrated that indoleacetic

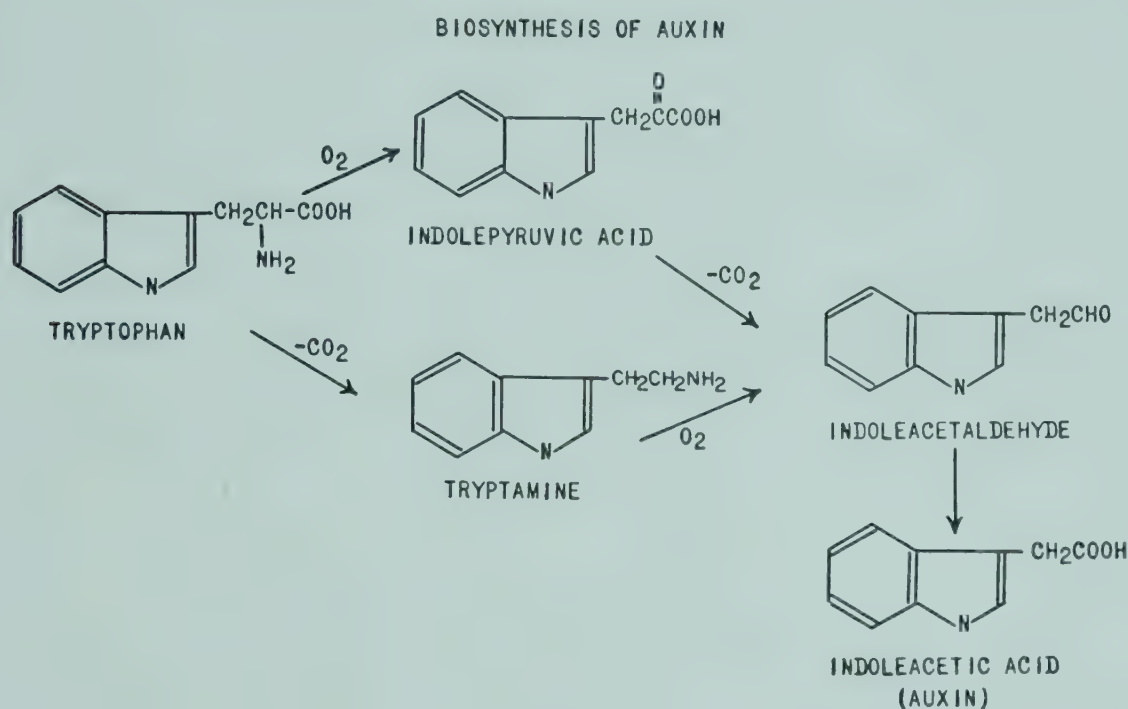


FIG. 4.

acid was the auxin produced by *Rhizopus*, and that its production depended on the presence of tryptophan in the medium (94). There have been numerous subsequent reports relating to the ability of various plants to produce auxin from tryptophan. The general metabolic significance of this relationship is broadened when it is recalled that the original identification of indoleacetic acid as a growth factor for plants was made by Kögl on material isolated from human urine (55).

Two types of reaction have been considered as reasonable mechanisms for the formation of indoleacetic acid from tryptophan: (1) decarboxylation to tryptamine, oxidation of the amine to indoleacetaldehyde, and oxidation of the aldehyde; or (2) oxidative deamination or transamination to form indolepyruvic acid, decarboxylation, and oxidation of the resulting aldehyde to indoleacetic



acid. There is evidence to support both postulations. Tryptamine has been reported to exist in *Acacia* (102), and is converted to indoleacetic acid by pineapple leaf preparations (29). Indolepyruvic acid is also active with pineapple leaf preparations (29), and in addition serves as a substrate for spinach leaves, which do not attack tryptamine (105). A corn smut which produces auxin from tryptophan was also reported to be inactive with tryptamine (107). Indolepyruvic acid has been identified by Stowe and Thimann in plant extracts (85), and the recent isolation of indoleacetic acid in human urine implies that the corresponding keto acid also occurs in humans (1).

There is considerable evidence that indoleacetaldehyde is the neutral substance with auxin properties which accumulates in plant material. This substance is thought to be indoleacetaldehyde because of its conversion to indoleacetic acid by an aldehyde oxidase, reaction with dimedon, reversible combination with bisulfite, and molecular size, determined by rate of diffusion through agar (63). This compound has recently been synthesized by Brown, Henbest, and Jones (10). The synthetic compound is both spontaneously and enzymatically converted to indoleacetic acid, and it seems that the acid is responsible for all of the biological activity of the preparation.

The two postulated pathways for indoleacetic acid formation both use tryptophan as the initial substrate and form indoleacetaldehyde, which has been shown to be an intermediate. As discussed above, there is evidence favoring the inclusion of both tryptamine and indolepyruvic acid routes, and it is possible that both exist. On the basis of known reactions, however, the pathway involving indolepyruvic acid may be considered more probable, since, as mentioned already, decarboxylation of tryptophan has not been demonstrated. Therefore the possibility must be considered that a branch occurs in the chain of reactions, and that indoleacetaldehyde formed from indolepyruvic acid can participate in a transamination to form tryptamine. Such a reaction might not be merely a blind alley, but might be followed by oxidation to the corresponding nitrile (Fig. 5). This compound has been isolated from cabbage leaves and found to be a more potent auxin than indoleacetic acid in at least one type



of assay (49). The conversion of indoleacetonitrile to the corresponding acid has been suggested, but the steps involved have not been elucidated. In addition to the obvious hydrolytic pathway, a reversal of the reactions outlined above, reduction to the amine,

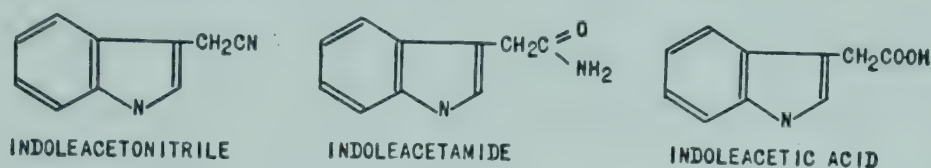


FIG. 5.

transamination, and oxidation of the aldehyde would give the same result. Such a mechanism would explain the inertness of a probable intermediate in the hydrolytic pathway, indoleacetamide (49).

A pea preparation was shown by Tang and Bonner (89) to oxidize indoleacetic acid with the consumption of one mole of oxygen and the production of one mole of carbon dioxide (Fig. 6). The product retained an indole ring. The product from a similar system obtained

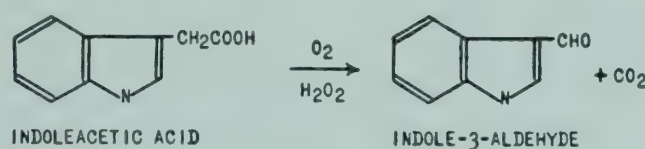


FIG. 6.

from beans was shown by Wagenknecht and Burris to retain an indole ring and to precipitate with 2,4-dinitrophenylhydrazine, and therefore was probably indole-3-aldehyde (100). Galston, Bonner, and Baker (25) have recently fractionated the pea system and have obtained evidence for the participation of an oxidase (possibly a flavoprotein) and a peroxidase. Horseradish peroxidase was also shown to attack indoleacetic acid, but the pea peroxidase preparation retained oxidase activity. The mechanism of indoleacetic acid oxidation has been studied extensively with inhibitors, whose roles have been discussed by Galston et al. (25). There are two oxidation steps in going from indoleacetic acid to indolealdehyde, and it is hoped that further purification of the enzymes involved will give more insight into the mechanism of this transformation.



## CONVERSION OF TRYPTOPHAN TO KYNURENINE

Many lines of investigation have brought investigators from various disciplines to studies relating to a series of reactions initiated by an oxidation of the indole ring of tryptophan. It would be desirable to acknowledge the contributions of many nutritionists, geneticists, and chemists in outlining these reactions, but in the interests of defining the current status of this area without excessive confusion, a chemical outline will be followed. It is hoped that some clarity in this presentation will be accepted as an excuse for the omission of proper acknowledgment of many significant studies.

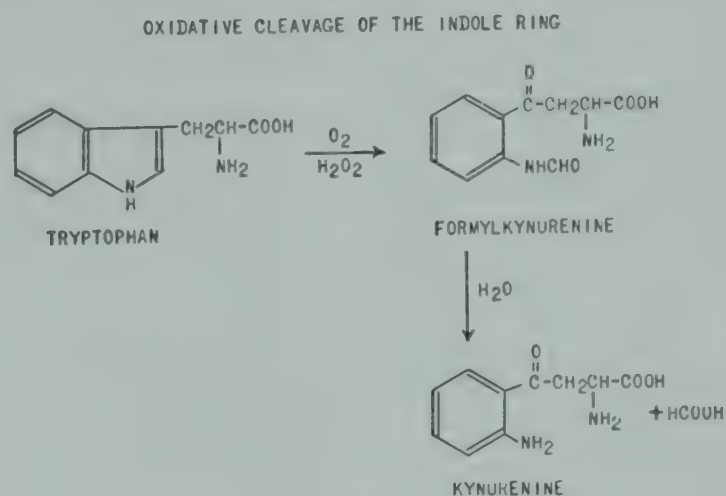


FIG. 7.

The oxidation of tryptophan to kynurenine through formylkynurenine is used to initiate a variety of biosyntheses (Fig. 7). The conversions indicated have been studied by a variety of methods, but most conveniently by spectrophotometric determinations, which detect all three compounds individually. While these reactions are not rapid in an absolute sense, even in crude extracts of liver, they are more rapid than subsequent steps, and therefore the initial tryptophan can usually be accounted for as the sum of these three compounds.

The mechanism of the initial oxidation is still incompletely understood. Studies by Knox and myself (53, 67) with mammalian liver enzymes have given some information about the nature of the oxidation. We found that a reaction specific for L-tryptophan requires both molecular oxygen and hydrogen peroxide, and that dyes cannot

serve as electron acceptor to any measurable extent. The need for hydrogen peroxide was shown by the pronounced inhibition of tryptophan oxidation by catalase, and the restoration of this oxidation by the addition of a system for producing hydrogen peroxide. A rather delicate balance between peroxide formation and utilization was required, since excess peroxide inactivates the system. For this reason, peroxide was generated enzymatically in a coupled reaction, instead of being added preformed. Crude liver systems contain both catalase and systems which form peroxide. Partial purification of the tryptophan-oxidizing system by precipitation of an active fraction at a slightly acid pH eliminated both of these influences, and permitted the demonstration of oxygen consumption as a direct consequence of tryptophan oxidation.

Among the outstanding questions is that of the number of enzymes involved. It has been shown that formylkynurenine accumulates as the product of tryptophan oxidation by the acid-precipitated enzyme. This represents two oxidative steps and ring opening. Since both oxygen and peroxide seem to be involved, it seemed reasonable to attribute one oxidation step to each. The failure of other oxidants to support any detectable reaction with tryptophan led to the suggestion that the initial reaction does not use oxygen, but requires peroxide, which is generated in the second step.

It is reasonable to propose individual enzymes for the catalysis of each of the oxidative steps. An alternative is suggested by recent studies involving various peroxidases. Theorell and Swedin (93) have found that horseradish peroxidase apparently acts as both an oxidase and peroxidase when dihydroxymaleic acid is used as a substrate, that this activity was inhibited by carbon monoxide, and that the inhibition was reversed by light. This evidence was taken to indicate that the iron atom of peroxidase existed in the ferrous form during this reaction, instead of remaining ferric, as is usual for peroxidases (92). The reported inhibition of the indoleacetic-oxidizing enzyme of peas by carbon monoxide is consistent with a similar behavior of this enzyme with its specific substrate. This viewpoint is strengthened by the observation reported by Galston et al. that the pea peroxidase retains some oxidase activity (25).



Recently, Knox has found that, unlike other peroxidases which attack tryptophan, the specific enzyme from liver is carbon-monoxide-sensitive (50). These three cases, then, are evidence for a general property of a group of enzymes: the ability to act as both oxidase and peroxidase with specific substrates. If this is the case, it might explain the inhibition of the tryptophan enzyme by excess hydrogen peroxide as being due to interference with the normal oxidation-reduction cycle of the iron in the enzyme.

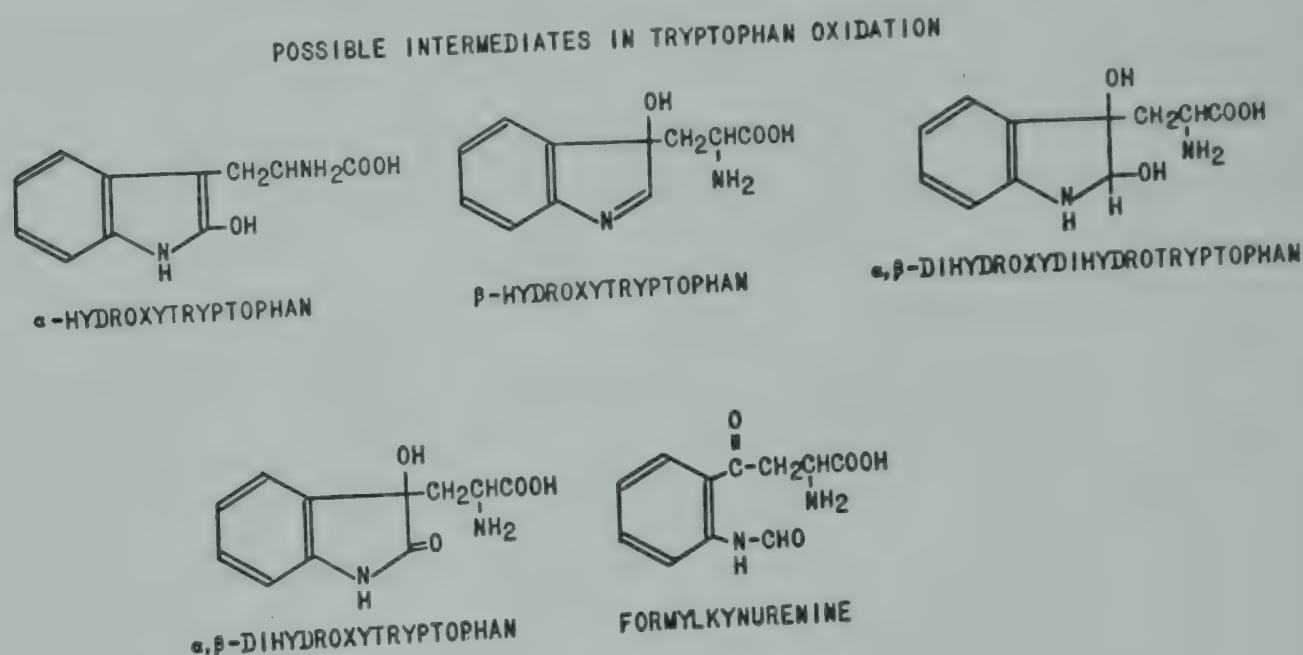


FIG. 8.

The nature of the oxidizing system will undoubtedly be clarified by further purification studies. Additional information may be anticipated from future syntheses of potential intermediates (Fig. 8). One suggested intermediate has been synthesized:  $\alpha$ -hydroxytryptophan (16, 56). This compound was implicated as an intermediate in the pathway leading to the formation of ommochrome, an insect eye pigment (13). Later work showed that the reaction found was an artifact caused by the spontaneous decomposition of  $\alpha$ -hydroxytryptophan to kynurenine, which is active in the assay used (18). The former compound has also been eliminated as a metabolite in a bacterial system which converts tryptophan to kynurenine (79). Hayaishi and Stanier have shown that the bacterial enzyme has properties very similar to those of mammalian liver preparations in the requirement for oxygen and peroxide (35). Both intact cells and cell-free extracts failed to attack synthetic  $\alpha$ -hydroxytryptophan.



Other possible intermediates between tryptophan and formylkynurenine are as yet unknown.  $\beta$ -Hydroxytryptophan,  $\alpha,\beta$ -dihydroxy-dihydrotryptophan, and  $\alpha,\beta$ -dihydroxytryptophan would all be of interest. The latter would be useful in attempting to learn whether the ring opens as a consequence of the oxidation, or whether a separate enzyme exists for rearranging the product of the oxidation steps.

Formylkynurenine is hydrolyzed by an enzyme found in mammalian liver (67), bacteria (35) and *Neurospora* (47). This was named simply formylase (67), and while the *Neurospora* enzyme was named more descriptively *kynurenine formamidase*, the more convenient, prior term will be retained for the present. This enzyme attacks a variety of formylated aromatic amines, but shows a decided preference for formylkynurenine among the analogs tested. The bacterial enzyme has been reported to be specific for L-kynurenine. Since the intact cells attack D-tryptophan, it was suggested that a mechanism exists for racemizing the original amino acid before oxidation begins. This could employ either a racemase such as that described for alanine (108) or the intermediate formation of indolepyruvic acid.

Two biological problems of general interest have been advanced through studies relating to the enzymes just discussed. Simultaneously and independently Suda, Hayaishi, and their collaborators in Japan (87) and Stanier and associates in this country (82, 84) studied the production of adaptive enzymes in bacteria. In this work, the degradation of tryptophan was studied by both groups. Although the organisms used accumulated different products, in each case support was found for the concept termed "successive adaptation" or "simultaneous adaptation." This concept, that adaptive organisms produce enzymes to attack not only the initial substrate but also all of the intermediates in its degradation, can be used to determine the inclusion of potential intermediates in a metabolic pathway, and to identify the enzymes involved. It has not only been applied to the further study of tryptophan metabolism, but has been found to be a generally useful tool.

Incidentally, in this case as in others already familiar, the adaptive



bacterial enzymes are found in much higher effective concentrations than the corresponding activities in other biological material. Besides the convenience thus afforded for the study of the enzymes themselves, their use in the preparation of unusual molecules is made practical, as Hayaishi showed for the preparation of kynurenine with extracts of tryptophan-adapted *Pseudomonas* (33).

The mammalian and bacterial enzymes are not identical. Both have been shown to be specific for L-tryptophan, and not to attack synthetic 5- or 7-hydroxytryptophan (21); but whereas Hayaishi will discuss later a potent inhibition of the bacterial enzyme by 5-hydroxytryptophan, this compound has very little influence on the reaction catalyzed by a preparation from rat liver.

A second result of the study of tryptophan oxidation was the discovery of the adaptive nature of this system in mammalian liver (54). Administration of tryptophan to an animal results in a gradual increase in the amount of enzyme activity found in the liver. The activity reaches a maximum of about 10 times the original amount in approximately 6 hours (for rats and rabbits), then after several hours gradually declines to normal. It was concluded from a study of the enzymes that this phenomenon represented a true enzyme synthesis, and this conclusion was supported by the findings of Lee and Williams (64) that ethionine inhibits the adaptive process and methionine reverses the ethionine effect; that is, ethionine presumably inhibits synthesis of the enzyme by competing with methionine, and excess methionine permits normal protein synthesis, and the formation of tryptophan-oxidizing enzymes to proceed. In contrast to bacterial adaptation, the oxidizing system which forms formyl-kynurenine is the only part of this pathway which has been found to change in the adaptive response in liver. Presumably in the intact organism this part of the sequence is limiting, and the overall rate of tryptophan oxidation would not be influenced by increases in the concentrations of other enzymes.

The nature of the adaptive response has been studied by Knox (51) and by Geschwind and Li (26). It is clear that one phenomenon involved is concerned with cortisone production in response to a pituitary signal, and that cortisone itself causes the liver to



produce the tryptophan-oxidizing system. This presumably is the mechanism by which a variety of aromatic compounds stimulates the production of the specific tryptophan enzyme. In addition, Knox finds that tryptophan continues to stimulate enzyme formation in adrenalectomized animals, which no longer respond to non-specific stimuli.

The importance of tryptophan oxidation in mammalian physiology is emphasized by this as yet unique reaction. However, it must be presumed that the dramatic effects of adrenal hormones involve other biological variations, and it may be that the value of the tryptophan system (which has been found only in liver) lies primarily in its example of the possibility of finding alterations at the enzyme level in response to pharmacological treatment. Several other enzymes have been found to vary in animals in various conditions, and it is probable that some of these alterations are analogous adaptations. Knox has recently reviewed this subject, and included a description of changes in the tryptophan system, both increase and decrease, in response to changes in the physiological state of the animal, for example, as modified with the progress of a tumor (51).

#### REACTIONS OF KYNURENINE

Genetic studies show that kynurenine is involved in both nicotinic acid (2) and ommochrome (12, 91) formation. Kynurenine and some of its derivatives undergo two types of reactions. The better-known kynureninase reaction (Fig. 9) is a hydrolysis to form anthranilic acid and alanine and has been shown to use pyridoxal phosphate as a cofactor (9, 36, 48, 57, 69, 106).

Kynureninase has been studied from several sources, mammals (52, 106), *Neurospora* (48), and bacteria (36). The basic mechanism of the splitting in each case closely resembles the others: pyridoxal phosphate is a cofactor for each, and the reported pH optima vary somewhat, but seem in general to be near pH 8. Each enzyme is specific for the L-form of kynurenine. The substrate affinities and specificities, however, vary greatly. Michaelis constants are reported as  $6 \times 10^{-6}$  for *Neurospora*,  $3.9 \times 10^{-5}$  for *Pseudo-*



*monas* and  $4 \times 10^{-4}$  for mammalian liver. The various enzymes differ in their relative abilities to attack kynurenine and 3-hydroxykynurenine (Table 1). Whereas the *Neurospora* enzyme was found to attack formylkynurenine, this is not a substrate for the bacterial

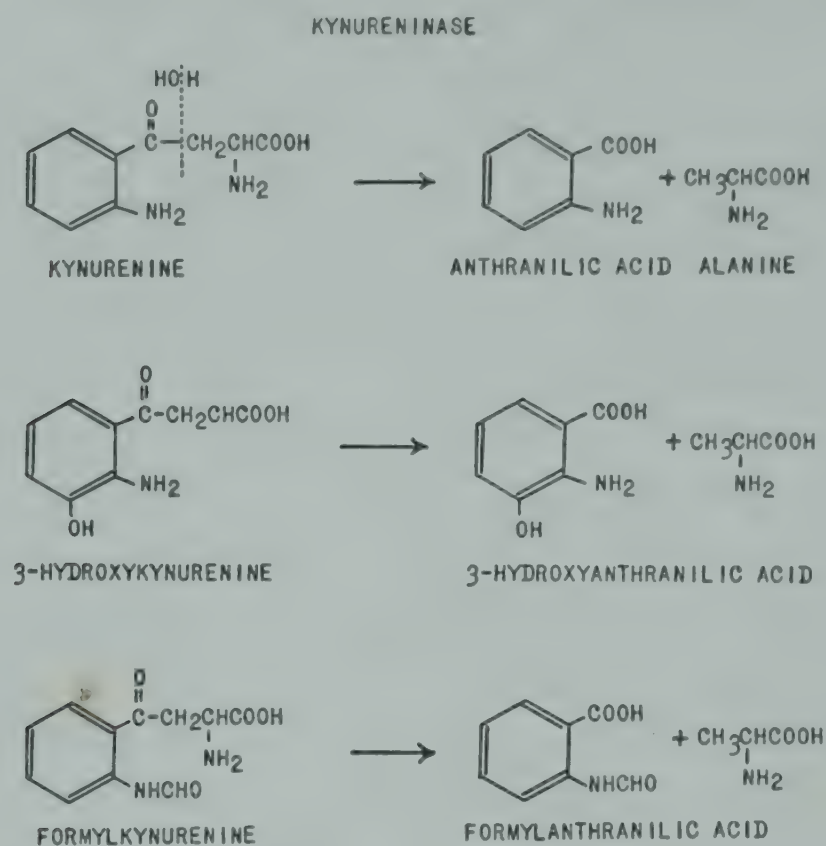


FIG. 9.

kynureninase. Hayaishi has surveyed other analogs of kynurenine with the bacterial enzyme, and has found interesting effects of modifications of the structure (32).

TABLE 1  
PROPERTIES OF KYNURENINASE

Source	Km	Kynurenine	
		3-OH-kynurenine	pH optimum
Liver	$4 \times 10^{-4}$	1	8.0
<i>Pseudomonas</i>	$3.9 \times 10^{-5}$	4.6	8.5
<i>Neurospora</i>	$6 \times 10^{-6}$	1	8.1

It is interesting to compare the kynureninase with the tryptophanase reaction; the splitting takes place between corresponding carbon atoms, but in one case a hydrogen and in the other a hydroxyl group is combined with the carbon  $\beta$  to the amino group. If it is

assumed that a Schiff's base is formed with the aldehyde of pyridoxal and the  $\alpha$ -amino group of the amino acid, it might be considered that the tautomeric shift of the double bond would reverse the polarity of the skeleton of the amino acid (Fig. 10). In the case of the

SCHIFF'S BASES OF PYRIDOXAL PHOSPHATE WITH  
TRYPTOPHAN AND KYNURENINE

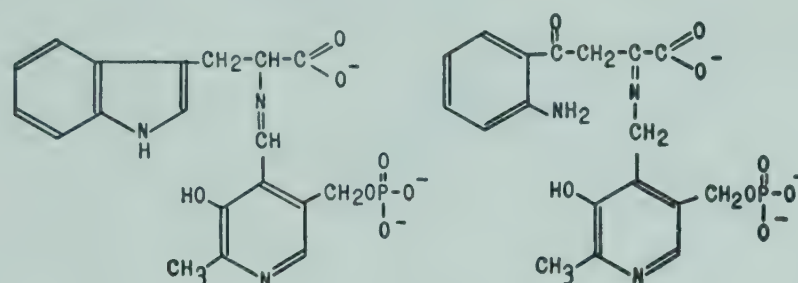


FIG. 10.

tryptophanase reaction, this would permit ejection of indole with an uptake of a proton at the carbon in question, either by hydrolysis to form the Schiff's base of serine, or by elimination, leaving an unsaturated chain of dehydroalanine. In the kynureninase reaction the tautomeric form of the Schiff's base is equivalent to a  $\beta$ -diketone, which would be expected to be hydrolyzed to form anthranilic acid. In either case, the reactive form would be determined by the still undefined binding to the specific protein.

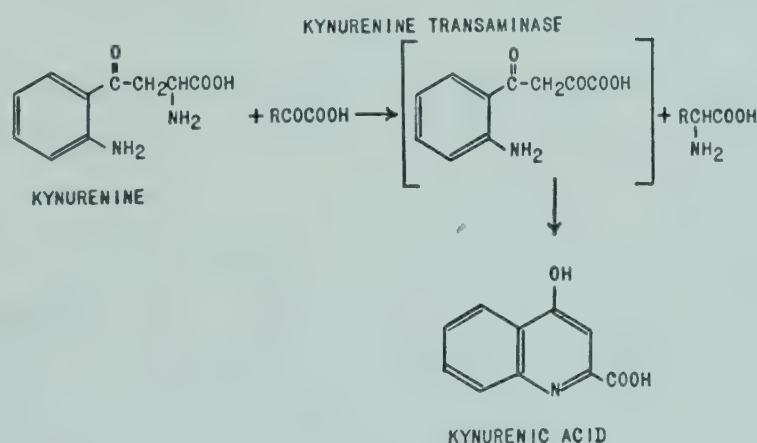


FIG. 11.

A second reaction of kynurenine has recently been dissociated from kynureninase. This is a transamination which presumably produces the corresponding  $\alpha$ -keto acid (Fig. 11). The synthesis of this compound has been suggested (66) but, in general, attempts to prepare it have yielded the cyclic Schiff's base. In enzyme systems only the



condensed quinoline derivatives are found. Kynurenine is thus converted to kynurenic acid, and 3-hydroxykynurenine in a similar manner becomes xanthurenic acid (Fig. 12), both of which are found in urine following tryptophan administration (17, 73). Jakoby (pers. commun.) has found both of these reactions to be catalyzed by a *Neurospora* transaminase, which requires pyridoxal phosphate.

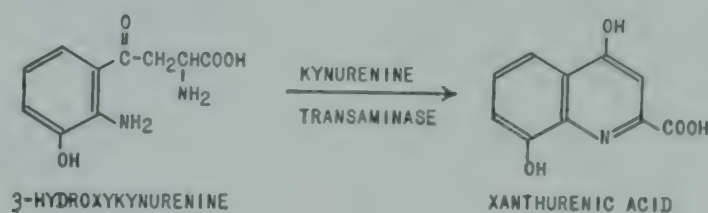


FIG. 12.

The repeated references to 3-hydroxykynurenine, made above, have anticipated the implication of this compound in several biosynthetic pathways. 3-Hydroxykynurenine was shown to accumulate as an intermediate in the formation of insect eye pigment (14). The synthetic compound (14) serves as a replacement for tryptophan or nicotinamide for certain *Neurospora* mutants (31). Tryptophan and metabolites derived from it have been shown to form nicotinic acid derivatives in whole animal and liver slice experiments (44, 113). Recently 3-hydroxykynurenine was shown to enter the nicotinic acid sequence of reactions in mammals (40). In certain pathological conditions in humans, this compound has been found to be excreted in the urine (19).

The mechanism by which 3-hydroxykynurenine is formed is completely unknown (Fig. 13). Schemes involving this compound usually indicate its derivation from kynurenine. The only basis for such a reaction is the fact that kynurenine will serve in some systems to replace the 3-hydroxy compound in reactions leading to the formation of 3-hydroxyanthranilic acid (or its products). As yet, no cell-free system has been reported to carry out the hydroxylation of kynurenine, and this is not from want of trying. An alternative suggestion was made by Dalglish (17). He and his associates, Knox and Neuberger, found the  $\alpha$ -N-acetyl derivatives of both kynurenine and 3-hydroxykynurenine in the urine of pyridoxine-

deficient rats given tryptophan (18). Acetylkynurenine had already been found in cultures of a *Neurospora* mutant (111). It was suggested that formation of an acetyl derivative might supply the substrate for hydroxylation and simultaneously deprive the blind-alley reactions leading to anthranilic and kynurenic acids of substrate.

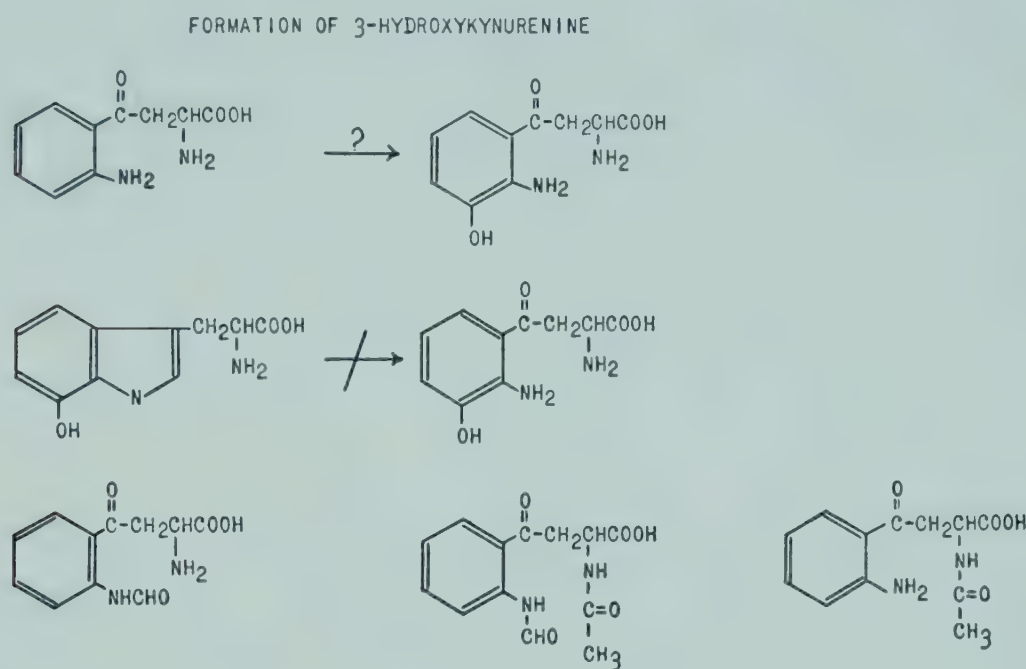


FIG. 13.

Another route for the synthesis of 3-hydroxykynurenine that is obvious on paper is the oxidation of 7-hydroxytryptophan. This compound was synthesized by Ek and Witkop (21) and was found by Hayaishi (32) to be inert in the bacterial tryptophan-oxidizing system, and also I have found that it was not metabolized at a measurable rate by rat liver preparations. Therefore, it must be concluded that the formation of that very important intermediate, 3-hydroxykynurenine, is an unknown process requiring either an enzyme which has resisted extraction or an untried substrate.

### 3-HYDROXYANTHRANILIC ACID

3-Hydroxykynurenine gives rise to 3-hydroxyanthranilic acid through the kynureninase reaction (Fig. 14). 3-Hydroxyanthranilic acid was shown to replace nicotinic acid for one *Neurospora* mutant and to accumulate as a product of tryptophan metabolism in cultures of another niacin-requiring mutant (6, 7, 71, 112). When given



tryptophan, a human tubercular patient was found to excrete 3-hydroxyanthranilic acid (74). This compound has also been shown to be converted to pyridine derivatives by rats (43, 72). The chemistry of this most intriguing conversion was outlined by isotope experiments. Heidelberger and collaborators showed that the side chain of tryptophan (which becomes the sidechain of kynurenine) is lost in the formation of nicotinic acid (38), and that the  $\beta$ -carbon of the indole ring becomes the carboxyl group of nicotinic acid (37).

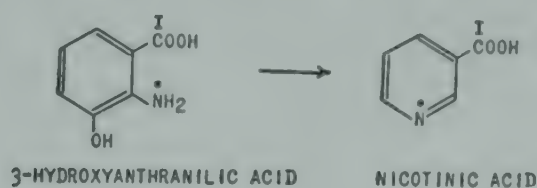


FIG. 14.

Schayer and Henderson showed that the indole nitrogen becomes a pyridine nitrogen in the rat (80). Similar isotope experiments with *Neurospora* showed that the indole nitrogen of tryptophan is the sole precursor of the pyridine nitrogen of niacin (77). Therefore, it has been concluded that the mechanism of nicotinic acid formation includes an opening of the ring of 3-hydroxyanthranilic acid and cyclization of the presumed intermediate amino aldehyde to form a pyridine ring.

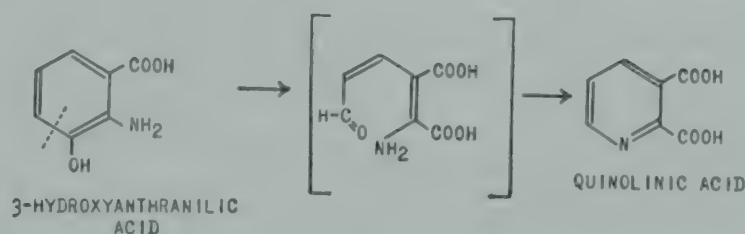


FIG. 15.

The site of ring splitting was indicated by Henderson, who showed that the structure of a product accumulated by *Neurospora* and excreted by rats was pyridine 2,3-dicarboxylic acid, or quinolinic acid (8, 41, 42) (Fig. 15). To obtain the adjacent carboxyls, it is necessary to postulate a 3,4 split in the anthranilic acid ring.

An obvious mechanism for the ring opening is one beginning with the formation of 3,4-dihydroxyanthranilic acid (Fig. 16). Makino et al. claim to have synthesized this compound and to have

found it equivalent to hydroxykynurenine in nicotinic acid formation by liver slices (65). The inclusion of this compound as an intermediate must be taken with reservation, for three reasons. First, 3,4-dihydroxykynurenine synthesized in Butenandt's laboratory was shown to be inert in both ommochrome- and niacin-forming systems (11), although it is attacked by kynureninase (39). Since the

## POSSIBLE INTERMEDIATES IN 3-HYDROXYANTHRANILIC ACID METABOLISM

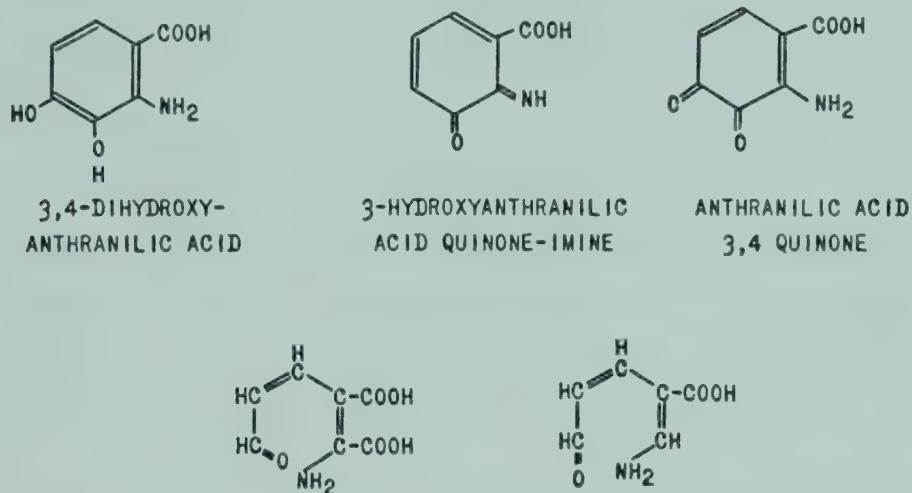


FIG. 16.

product of kynureninase splitting must be the corresponding 3,4-dihydroxyanthranilic acid, this compound cannot be an intermediate. Second, the experiments of Makino et al. were carried out with 24-hour incubations, during which spontaneous reactions could have altered the substrates. Third, samples sent by Makino to investigators in this country were inactive in the assays used, and his experiments could not be confirmed (42a).

The mechanism of 3-hydroxyanthranilic acid conversion to nicotinic acid is under investigation in several laboratories. Bokman and Schweigert found that a soluble extract from rat liver acetone powder would oxidize 3-hydroxyanthranilic acid quantitatively to quinolinic acid (5). They reported that in this reaction an intermediate accumulates which can be measured spectrophotometrically and exhibits an absorption maximum at 360  $m\mu$ . I have recently estimated the extinction coefficient of the intermediate to be approximately 30,000. Henderson reported that the enzyme which forms the intermediate requires ferrous ions and is inhibited by  $\alpha,\alpha'$ -dipyridyl (40). The inhibition has been confirmed in Schweigert's laboratory and by



myself. There is disagreement about the sensitivity of this enzyme to cyanide. Schweigert and Henderson reported the system to be insensitive, but Viollier and Süllman found inhibition by cyanide in their manometric determinations with a particulate enzyme (99), and I find a marked inhibition at  $10^{-5}$  M., but not a complete inhibition even at  $10^{-2}$  M., when using a spectrophotometric assay with extracts of acetone powder.

The nature of the product is still obscure. In spite of the difficulties mentioned below, Henderson (pers. commun.) believes that one mole of oxygen is consumed in its formation. This material is very unstable in neutral and alkaline solutions; eventually a quantitative yield of quinolinic acid is found. In acid the intermediate is destroyed immediately, and gives a product which Schweigert and collaborators (pers. commun.) have purified and found to be free from nitrogen, and therefore a degradation product of the intermediate.

In concentrated solutions of 3-hydroxyanthranilic acid the intermediate does not accumulate to a proportional extent, and a red-brown pigment, first noted by Viollier and Süllman (99), accumulates. This may be related to ommochrome. There is no evidence that the color formation from the intermediate is enzyme-catalyzed. In addition to pigment formation, another complication in the study of nicotinic acid synthesis is introduced by high substrate concentration. The reaction is inhibited, apparently by the intermediate, at concentrations which are so low as to limit accumulation of material for isolation and to make oxygen consumption difficult to measure. The net reaction of 3-hydroxyanthranilic acid to either quinolinic acid or to nicotinic acid plus carbon dioxide involves two oxidation steps. These are not comparable to the two-step oxidase-peroxidase reactions discussed above. Catalase has no effect on either the formation or destruction of the 360  $m\mu$  absorption.  $H_2O_2$  has no effect on either the spontaneous or enzymatic disappearance of the intermediate, but completely inactivates the enzyme which attacks 3-hydroxyanthranilic acid.

The acetone powder extracts of liver, as used by other workers,



do not contain detectable amounts of enzyme for the further metabolism of the intermediate. Fresh or frozen liver, on the other hand, can be extracted to yield a soluble protein which catalyzes the removal of the 360 m $\mu$  absorption. This enzyme is completely inhibited by cyanide. Experiments are in progress to determine the nature of the reaction and its product. Until such information is available, the position of quinolinic acid as intermediate in niacin formation must remain in doubt. Quinolinic acid has been shown by several investigators to be an inefficient precursor of nicotinic acid, and may be, as was suggested by others, a side-product. This could easily arise by the non-enzymatic reaction of the intermediate in the absence of sufficient enzyme for its conversion to niacin. The activity of the enzyme which causes the accumulation of the intermediate is fast compared with most of the other known enzymes involved in the metabolism of tryptophan.

At the present time several structures may be considered as intermediates leading to nicotinic acid formation, but none can be accepted or rejected on the basis of present knowledge.

### THE AROMATIC PATHWAY

Certain strains of *Pseudomonas* use tryptophan as a major source of carbon, nitrogen, and energy (83, 87). In these organisms, the oxidation of tryptophan to kynurenine proceeds as described above, and the hydrolysis of kynurenine to anthranilic acid and alanine is carried out by kynureninase. The subsequent oxidation of anthranilic acid has been partially elucidated (Fig. 17), and been shown to result in the formation successively of catechol, *cis,cis*-muconic acid, and  $\beta$ -ketoadipic acid.

The oxidation of anthranilic acid to catechol has not been achieved in cell-free systems, but the position of catechol in this sequence was established by means of the successive adaptation technique. The adaptive enzyme which oxidizes catechol to *cis,cis*-muconic acid has been extracted successfully by workers in Japan and named pyrocatechase (34). This enzyme was purified from extracts of acetone-dried adapted cells. The purified enzyme could be shown to require



ferrous iron (86). The subsequent hydration to  $\beta$ -ketoadipic acid also occurs with cell-free extracts.

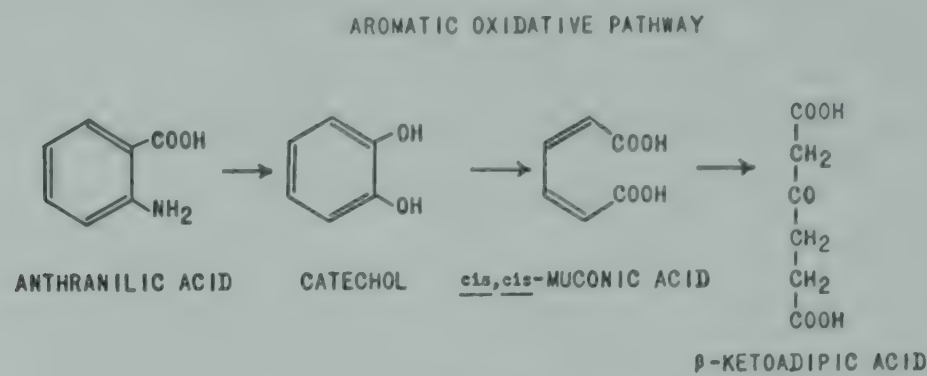


FIG. 17.

SUMMARY

The principal pathways of tryptophan metabolism are illustrated on the accompanying diagram (Fig. 18). It is unfortunate that

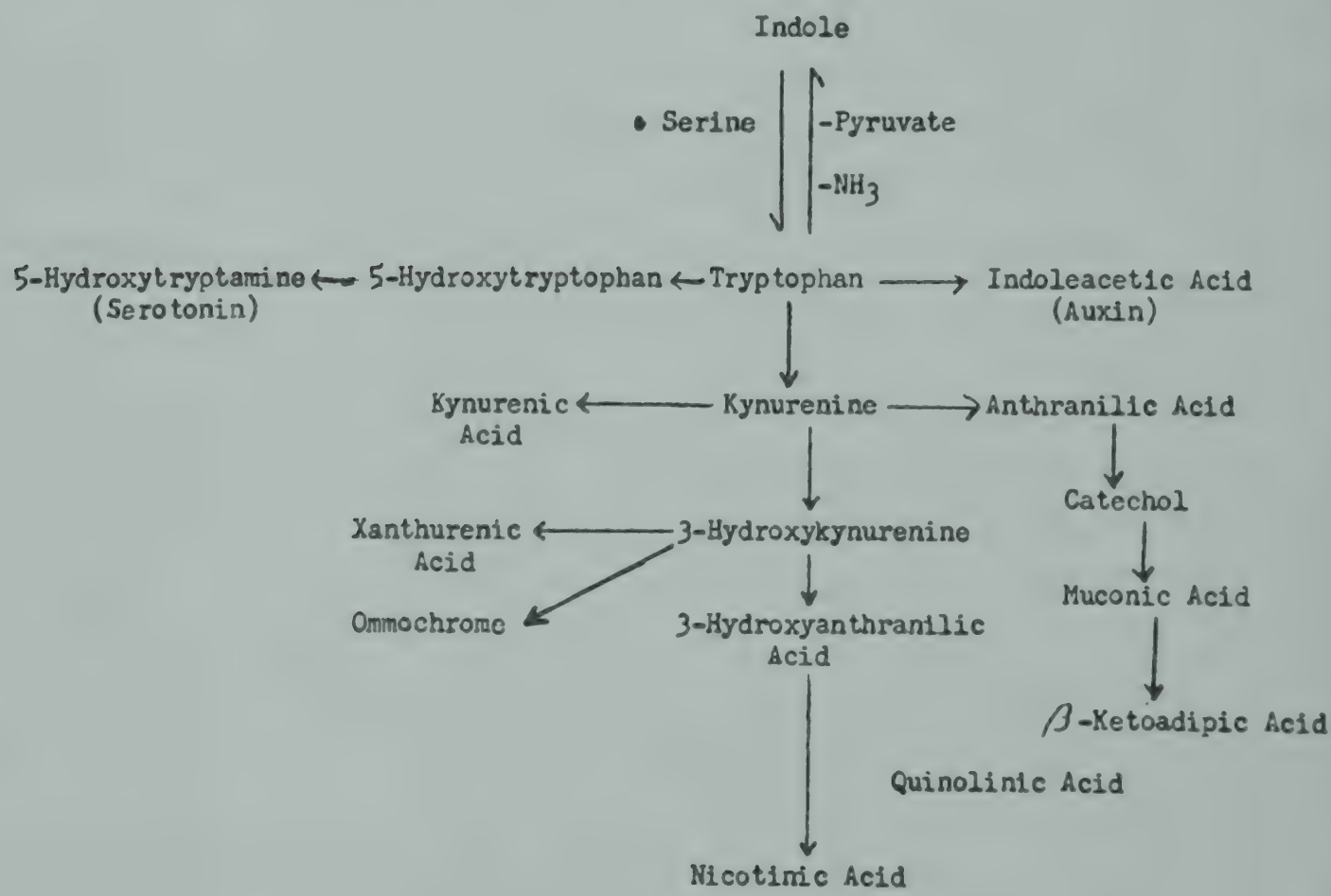


FIG. 18.

more information cannot be included about other reactions which must occur in biological systems, such as the formation of methyl-tryptophan (abrine) (46) and the synthesis of phalloidin, the mushroom toxin which contains tryptophan substituted on the 2 position

of the indole ring (104). The metabolism of D-tryptophan involves as yet unknown pathways leading to the formation of D-kynurenine (62). Both kidney L- and D-amino acid oxidase attack tryptophan (4, 58), presumably with the formation of indolepyruvic acid, which may be further oxidized to indoleacetic acid. Indolepyruvic acid may also be formed by transamination, as reviewed earlier in this symposium by Meister. The formation of gramine (dimethyl indole methyl amine) from tryptophan was recently shown in barley sprouts (64a). The formation of skatole (5-methylindole) from tryptophan was demonstrated in 1904 (45), but the biochemical mechanism has not yet been discovered. It should be noted that in addition to these incompletely discussed problems, the other reaction sequences discussed were arbitrarily stopped at convenient places. Many of the products shown as end-products are already known to be metabolized further by various organisms.

The reactions shown include the formation of the animal hormone serotonin, the plant hormone indoleacetic acid, and the vitamin nicotinic acid, which is a constituent of the pyridine nucleotides so essential for biochemists and other organisms. In the syntheses of these compounds many problems remain to be solved in order that the reaction sequences and the nature of the enzymatic reactions can be known. The wealth of information already obtained and the exciting extensions into related physiological fields resulting from past studies are undoubtedly optimistic indications of the rewards to come from studies of this amino acid in the future.

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# AN INTERRELATIONSHIP BETWEEN TRYPTOPHAN, TYROSINE, AND PHENYLALANINE IN NEUROSPORA

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DURING THE COURSE of study of kynureninase from *Neurospora* (1, 2), it was found that the amount of kynureninase extractable from mycelium could be increased as much as several hundred-fold by growth on kynurenine or kynurenine precursors. The finding of the adaptive nature of kynureninase formation suggested the utilization of this phenomenon as a tool for the investigation of precursors of kynurenine. Thus, a compound which gives rise to kynurenine would be expected to increase the quantity of kynureninase in the mycelium.

Such an increase has been found for the known kynurenine precursors (tryptophan, indole, anthranilic acid) as well as for phenylalanine and tyrosine. Representative data are shown in Table 1. Before postulating that the last-mentioned aromatic amino acids are precursors of tryptophan, however, the question arises as to whether other mechanisms are able to account for the increase in enzyme content when growth takes place in the presence of phenylalanine or tyrosine. An examination of the experimental method is also required.

Mycelium was produced in stationary, 2-liter Fernbach flasks containing 333 ml. of *Neurospora* minimal medium (3), supplemented as desired. A wild-type strain (5256 A) of *Neurospora crassa* was used. The formed mycelium was extracted and assayed as previously

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TABLE 1  
INDUCTION OF KYNURENINASE

Supplement ( $4 \times 10^{-4}$ M.)	S.A. <sup>1</sup>
No additions	6.4 <sup>2</sup>
L-kynurenine	61
L-tryptophan	685
indole	400
anthranilic acid	44
L-phenylalanine	23
L-tyrosine	11
shikimic acid	5.2
quinic acid	6.8

<sup>1</sup> S.A. = specific activity as defined in the text.

<sup>2</sup> An S.A. of 6.4 is the highest activity ever obtained in minimal medium in approximately 50 trials under various conditions and stages of growth.

described (1). The specific activity (abbreviated: S.A.) of the enzyme is defined as units of activity per mg. of protein. One unit is equal to 1  $m\mu M$ . of anthranilic acid formed from L-kynurenine in 40 minutes under standard conditions (1).

Experimentally the criterion which was used to judge an increase in kynureninase content was the greater specific activity of the extracted enzyme. If, in such an experiment, an increase in specific activity is not found a conclusion as to the precursor function of a compound may not be reached. For example, a compound many steps removed from kynurenine may not yield the adaptive response. Indeed, shikimic acid, which has been considered to be a precursor of several aromatic compounds, including tryptophan and therefore kynurenine, did not produce a significant increase in the specific activity of the isolated kynureninase. In this case it may be argued that an enzyme involved in the conversion of shikimic acid to kynurenine is already operating at its maximum "capacity," or that, because of the many reactions which shikimic acid and its products may undergo, a relatively small fraction of the added compound is directed toward the formation of kynurenine.

It should be noted that kynurenine, a substrate of kynureninase, was not as efficacious for elicitation of the enzyme as was tryptophan. It can be seen from Table 1 that kynurenine yields approximately the same S.A. as does anthranilic acid. One explanation of this finding depends on the formation of anthranilic acid from kynurenine early in the growth of the organism and, indeed, the early formation of anthranilic acid under these circumstances has been described (4). It may be argued that although the S.A. is very high while kynurenine is present, i. e., in very young mycelia, as growth proceeds and anthranilic acid is substituted for kynurenine in the medium, the S.A. value would drop to that achieved by anthranilic acid alone.

That the pathways of biosynthesis of phenylalanine, tyrosine, *p*-aminobenzoic acid, and tryptophan are related and that shikimic acid is thought to be a precursor of all of these compounds has been amply documented (5, 6). Work on the reversibility of these reactions is, however, sparse. *Neurospora* mutants have been found which, although they require tryptophan or nicotinic acid for growth, could have this growth requirement satisfied by phenylalanine or by tyrosine (4, 7, 8). In a series of experiments which are similar to the ones reported here, Knox (4) has found an adaptive enzyme in rats, tryptophan peroxidase, which is increased by tryptophan and to a lesser extent by phenylalanine or tyrosine. In the present work, the addition of either phenylalanine or tyrosine to *Neurospora* growth medium was found to give rise to a significant increase in the quantity and specific activity of kynureninase. In the case of both the growth (4, 8) and the enzyme adaptation experiments with *Neurospora*, *p*-aminobenzoic acid was inactive.

One cannot definitely ascribe the inductive effect of the two aromatic amino acids to their conversion to kynurenine. Two other possibilities appear particularly promising in accounting for the experimental observations. The two amino acids could, for example, serve as heterologous inducers of kynureninase. Another alternative is that these compounds exert a sparing effect on the tryptophan requirement.

Possibly the best evidence against heterologous induction is the



absence of an adaptive response when mutants requiring phenylalanine or tyrosine are supplemented with an excess of the respective amino acid. A number of analogues of the known intermediates in tryptophan synthesis have also been examined without the demonstration of one which could induce heterologously.

Phenylalanine and tyrosine may exert a sparing effect on the tryptophan requirement in that the addition of the compounds relieves the organism of the manufacture of phenylalanine or tyrosine, thereby increasing the amount of precursor available for tryptophan formation. Such sparing effects have been noted and remain a possibility (10-12). It would seem, however, that the lack of the adaptive response in the case of the mutants requiring the two aromatic amino acids speaks against a sparing effect mechanism (Table 2). That the mutants retain the ability to adapt is shown by an experiment in which tryptophan was also included in the growth medium of a phenylalanine-requiring mutant (Table 2).

TABLE 2  
KYNURENINASE ADAPTATION BY MUTANT STRAINS

Strain	Requirement	Supplement	Concentration	S.A.
16329A	phenylalanine	L-phenylalanine	10	4.3
"	"	L-phenylalanine and L-tryptophan	0.1 and 4	97
6974A	tyrosine	L-tyrosine	10	1.7

If a sparing effect were operative such mutants, being relieved of the necessity of producing these amino acids, might channel a common precursor in the direction of kynurenine and so lead to the induction of kynureninase. The finding that adaptation does not occur under such conditions has been demonstrated.

The mechanism by which phenylalanine and tyrosine induce kynureninase formation is, obviously, not well established. The data suggest however, that these two amino acids are converted to an intermediate in the synthesis of kynurenine, and thereby give rise to an increase in the concentration of kynurenine leading to a subsequent increase in kynureninase. The fact that phenylala-

nine and tyrosine mutants, when grown in the presence of an excess of the required amino acid, do not yield an adaptive response leads to the further suggestion that the enzymatic step which is blocked in each of these mutants is a reversible one, and that the blocked enzyme is one involved in converting the amino acid to some intermediate in the synthesis of kynurenine. Lacking pertinent data, a discussion of the exact mechanism which is involved in the shunting of the amino acids to tryptophan synthesis must be reserved.

It must be stressed that the evidence which has been presented bears only on the relationship of phenylalanine and tyrosine to tryptophan synthesis under the "abnormal" conditions in which phenylalanine or tyrosine are present in vast excess of the organism's requirements.

It is a pleasure to acknowledge the interest of Dr. D. M. Bonner during this study.

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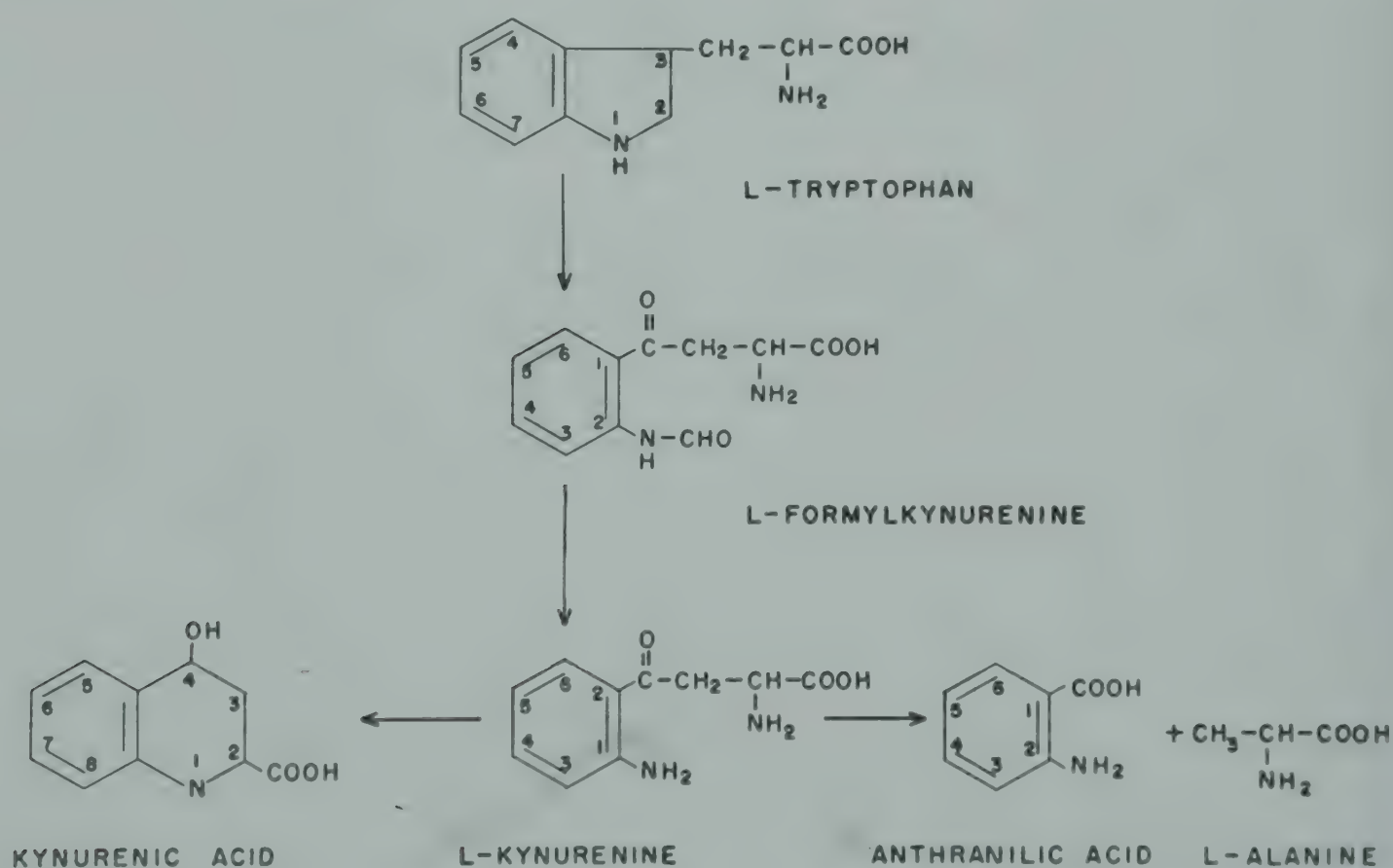


# ENZYMATIC STUDIES ON THE METABOLIC INTER-RELATIONSHIP OF HYDROXY-SUBSTITUTED DERIVATIVES OF TRYPTOPHAN AND ITS INTERMEDIATE METABOLITES \*

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RECENT STUDIES of tryptophan metabolism have established that the following scheme represents two major metabolic pathways of tryptophan in mammals, insects, molds, and bacteria (for a general review see 1 and 2).



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A number of hydroxy-substituted derivatives of tryptophan and its intermediate metabolites have been found in nature and some of them are shown to be metabolically related to, or identical with, substances which exhibit specific biologic activities. Among these, 2-hydroxytryptophan was probably the first of them to be isolated from a natural source (3) and was postulated to be an intermediate substance between tryptophan and kynurenine (4, 5). Later this was disproved by experiments with synthetic 2-hydroxytryptophan (6, 7, 8). Two others, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, were shown to be precursors of nicotinic acid (9, 10). A corresponding hydroxy-derivative of kynurenic acid (xanthurenic acid or 8-hydroxykynurenic acid), first isolated by Musajo (11) from the urine of rats, was recently claimed to have diabetogenic activity (12). Although Wiss (13) reported that animal kynureninase hydrolyzes 3-hydroxykynurenine about twice as fast as kynurenine and converts the former to 3-hydroxyanthranilic acid and alanine, it has not been established whether kynurenine is directly oxidized to form 3-hydroxykynurenine or whether hydroxylation occurs at the tryptophan level and the oxidation product, 7-hydroxytryptophan, may be further oxidized to yield 3-hydroxykynurenine by way of 3-hydroxyformylkynurenine. Hydroxylation at the tryptophan stage has in fact been suggested as a possibility (14), but experimental evidence is lacking. Recent studies (15) indicate that 5-hydroxytryptophan is a precursor of 5-hydroxytryptamine, which is a vaso-pressor substance and has been found in serum (16), intestinal mucosa (17), blood platelets (18), and also in the parotid glands of *Bufo marinus* (19). A corresponding derivative of anthranilic acid (5-hydroxyanthranilic acid) was isolated from the urine of rats (20) which were given a large amount of anthranilic acid. The corresponding derivatives of kynurenine (5-hydroxykynurenine) and kynurenic acid (6-hydroxykynurenic acid) have not been reported from natural sources, but chemical synthesis of the former compound was recently reported by three different laboratories (21, 22, 23). Since the search for the enzyme system which carries out hydroxylation of the benzene ring of these aromatic compounds has so far been



unsuccessful, it has not been established at what stage of tryptophan metabolism the hydroxylation takes place.

The present investigation was undertaken to examine the specificity of the tryptophan peroxidase-oxidase system, of kynureninase, and of kynurenine transaminase in order to study the possible metabolic relationships of these hydroxy-substituted derivatives of tryptophan and its intermediate metabolites. Tryptophan peroxidase-oxidase

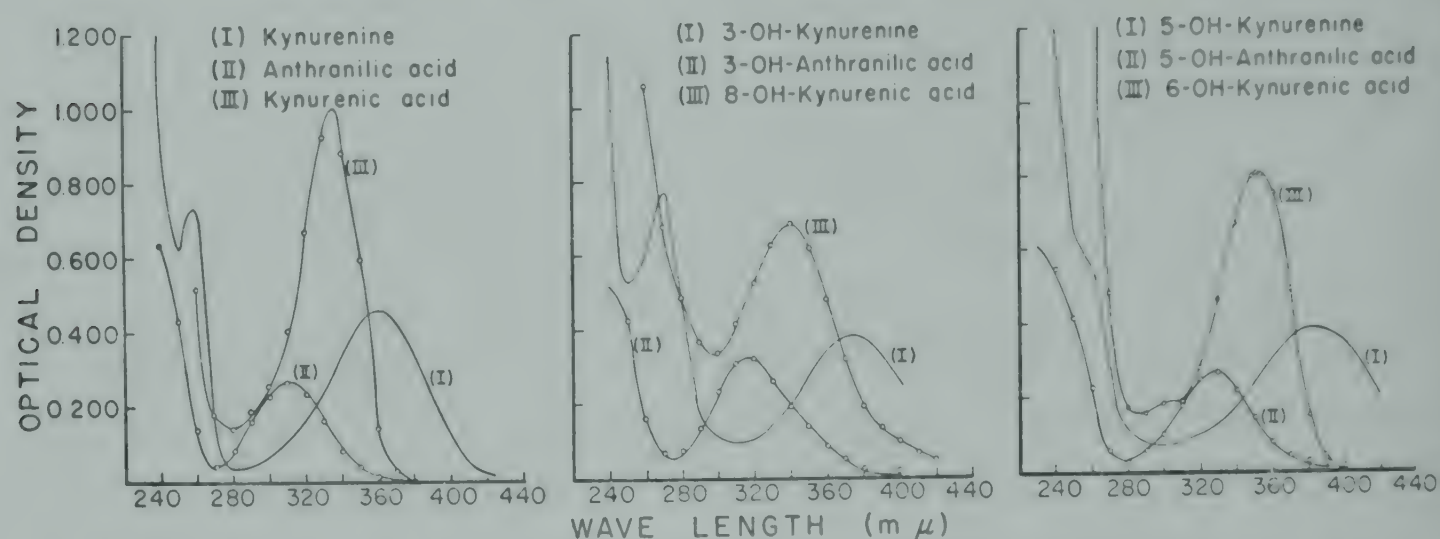


FIG. 1. Spectra of kynurenine, anthranilic acid, kynurenic acid, and their hydroxylated derivatives. The concentration of each compound is  $10^{-4}$  M. The pH was adjusted at 7.0 with 0.02 M. phosphate buffer. Continuous line: authentic samples. Circles, eluted samples.

TABLE 1

$R_f$  values

	Butanol, water acetic acid (5:4:1)	80% propanol	Fluorescence
Kynurenine			
Unsubstituted	0.40	0.28	Pale blue
3-hydroxy	0.36	0.25	Greenish yellow
5-hydroxy	0.28	0.16	Intense yellow
Anthranilic acid			
Unsubstituted	0.88	0.85	Purple
3-hydroxy	0.84	0.70	Blue
5-hydroxy	0.58	0.61	Whitish blue
Kynurenic acid			
Unsubstituted	0.51	0.33	Dark blue
8-hydroxy	0.44	0.37	Blue
6-hydroxy	0.40	0.20	Whitish pink

(24) and kynureninase (25) were prepared from tryptophan-adapted cells of *Pseudomonas* strain 23, and kynurenine transaminase (26) from tryptophan-adapted cells of *Pseudomonas* strain 6, as described before, since bacterial enzymes are much more active than the same enzymes of animal origin. Manometric and spectrophotometric experiments were carried out as previously described (24, 25). The spectra and the  $R_f$  values, as well as the fluorescence on paper chromatographs of the substrates, are presented in Fig. 1 and Table 1.

### THE SPECIFICITY OF TRYPTOPHAN PEROXIDASE-OXIDASE

Two micromoles of DL-5- and DL-7-hydroxytryptophan<sup>1</sup> were incubated with 1 ml. of cell-free extracts of tryptophan-grown *Pseudomonas* strain 23, buffered at pH 7.0 at 30° C. No oxygen uptake was observed above the endogenous level even after the incubation was carried out for more than one hour, while, under identical conditions, 2 micromoles of L-tryptophan were metabolized within 15 minutes and were converted to  $\beta$ -ketoadipic acid, as reported previously (24) (Table 2). In addition to no oxygen uptake, further

TABLE 2

Substrate						
L-tryptophan	—	+	—	—	+	+
DL-5-hydroxytryptophan	—	—	+	—	+	—
DL-7-hydroxytryptophan	—	—	—	+	—	+
Oxygen uptake (microliters per 30 minutes)	32	132	31	34	48	84

Each Warburg vessel contained 2  $\mu$ M. of substrates as indicated above, 1.0 ml. of *Pseudomonas* extracts (12 mg. protein), 50  $\mu$ M. of phosphate buffer, pH 7.0, in a total volume of 1.8 ml. 0.2 ml. of 2 N KOH in the center well. Temperature 30° C. Gas phase, air.

evidence was provided by spectrophotometric examination of the deproteinized supernatant fluid of the reaction mixture and by paper chromatographic analyses. No appreciable change was detected with the two hydroxytryptophans either spectrophotometrically or chro-

<sup>1</sup> 5- and 7-hydroxytryptophan were kindly furnished by Dr. B. Witkop (27).



matographically when compared with the boiled enzyme control. When the oxidation of tryptophan was tested in the presence of either 5- or 7-hydroxytryptophan, approximately a 95 per cent and a 50 per cent inhibition, respectively, were observed. As will be discussed later, this inhibition is shown to be of a competitive nature and not to be due to impurities present in the preparation of the hydroxytryptophans.

These data clearly demonstrate that the tryptophan peroxidase-oxidase system does not detectably oxidize 5- or 7-hydroxytryptophan to the corresponding kynurenine derivatives and, together with previous observations that 2-hydroxy- and 4-, 5-, and 7-methyl-tryptophans are not attacked by this enzyme (24), provide further proof for the rigorous specificity of this enzyme system. Similar experiments with 5- and 7-hydroxytryptophan conducted by Mehler with the animal enzyme have also shown negative results (28).

Since this extract was shown to convert tryptophan to  $\beta$ -ketoadipic acid by way of formylkynurenine, kynurenine, anthranilic acid, catechol, and *cis-cis* muconic acid (24), the exact site of inhibition of tryptophan oxidation by hydroxytryptophans has to be determined, although a competitive type of inhibition at the primary step was suspected to be most likely. In order to study the exact site and type of inhibition, a direct spectrophotometric assay was developed for accurate determination of the activity of the tryptophan peroxidase-oxidase system. Cell-free extracts of *Pseudomonas* No. 6 were used as an enzyme source, since these extracts convert almost 90 per cent of tryptophan to kynurenine (29). The formation of formylkynurenine was determined by the increase in optical density at 332  $m\mu$  because tryptophan does not absorb at this wave length, while the molecular extinction coefficient ( $\Sigma = 2,600 \text{ cm}^2/\text{mole}$  at pH 7.0) of formylkynurenine and kynurenine are almost identical at this point. This method permits the use of a high concentration of substrate and also the accurate measurement of the rate of the primary reaction (tryptophan  $\rightarrow$  formylkynurenine), despite the presence of the second reaction (formylkynurenine  $\rightarrow$  kynurenine). With this assay method, the formation of formylkynurenine from



L-tryptophan was shown to be inhibited almost completely with the equivalent amount of DL-5-hydroxytryptophan. Under the same conditions, 7-hydroxytryptophan is about 55 per cent inhibitory (Table 3). Since 5-methyltryptophan<sup>2</sup> has been known to interfere with either synthesis or utilization of tryptophan in some unknown way

TABLE 3  
INHIBITION OF ENZYMATIC OXIDATION OF TRYPTOPHAN

Inhibitor	None	5-OH-tryptophan	7-OH-tryptophan	5-CH <sub>3</sub> -tryptophan
m $\mu$ M. of formyl-kynurenine formed per minute	42.5	1.1	19.4	37.2

The assay system contains, in 1.0 ml., 1.0  $\mu$ M. of L-tryptophan, 1.0  $\mu$ M. of inhibitors (DL form) as indicated, 5  $\mu$ M. of phosphate buffer, pH 7.0, and *Pseudomonas* extract (0.5 mg. of protein).

(30, 31, 32) its action in respect to this enzyme system was tested together with 4-, 6-, and 7-methyltryptophans. Under the same conditions, 5-methyltryptophan showed a 12 per cent inhibition, but the other monomethyltryptophans were less inhibitory.

The affinity of the enzyme to the substrate and the inhibitors was determined according to the method of Lineweaver and Burk (33). From the data shown in Fig. 2, the inhibition appears to be of a competitive nature. The dissociation constant of the enzyme-inhibitor complex ( $K_i$ ) was calculated to be approximately  $2.0 \times 10^{-6}$  M. for 5-hydroxytryptophan and  $1.2 \times 10^{-4}$  M. for 7-hydroxytryptophan, assuming that only the L-isomer is the inhibitory form. Since the dissociation constant ( $K_s$ ) of L-tryptophan is approximately  $4.0 \times 10^{-4}$  M, 5-hydroxytryptophan has about a 200-fold affinity to the enzyme as compared to its substrate. Since 5-hydroxytryptophan was shown to be a precursor of serotonin and appears to be a normal metabolite in mammals, its antimetabolite activity may have some significance in regulating the oxidative metabolism of tryptophan in vivo.

<sup>2</sup> 4-, 5-, 6-, and 7-methyltryptophan were synthesized by Dr. H. N. Rydon and were kindly supplied through the courtesy of Dr. R. Y. Stanier.



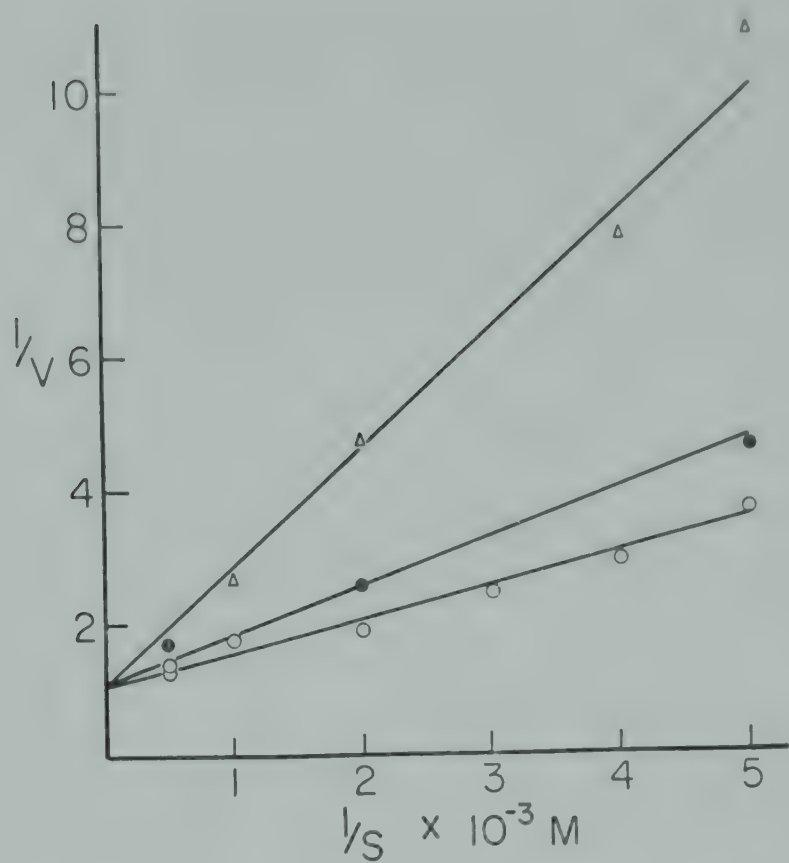


FIG. 2. Rate of the tryptophan oxidation in relation to substrate and inhibitor concentration.

The assay system (1.0 ml.) contained 5  $\mu$ M. of phosphate buffer, pH 7.0, and *Pseudomonas* extracts (0.5 mg. of protein). Open circles, L-tryptophan only. Solid circles, in the presence of 0.1  $\mu$ M. of DL-7-hydroxytryptophan. Triangles, in the presence of 0.01  $\mu$ M. of DL-5-hydroxytryptophan.

TABLE 4

INHIBITION OF GROWTH OF *E. coli* BY 5-HYDROXY- AND 5-METHYL-TRYPTOPHAN

	Molar Concentration of Inhibitors					
	$6.4 \times 10^{-5}$	$3.2 \times 10^{-5}$	$1.6 \times 10^{-5}$	$8 \times 10^{-6}$	$4 \times 10^{-6}$	0
DL-5-hydroxytryptophan	+	+	++	+++	++++	++++
DL-5-hydroxytryptophan in the presence of $5 \times 10^{-4}$ M. L-tryptophan	++++	++++	++++	++++	++++	++++
DL-5-methyltryptophan	±	±	+	++	+++	++++
DL-5 methyltrvptophan in the presence of $5 \times 10^{-4}$ M. L-tryptophan	++++	++++	++++	++++	++++	++++

The mediuc contains 0.15%  $\text{KH}_2\text{PO}_4$ , 1.35%  $\text{Na}_2\text{HPO}_4$ , 0.02%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2%  $\text{NH}_4\text{Cl}$ , 0.001%  $\text{CaCl}_2$ , 0.00005%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.4% glucose. Incubation was carried out at 27° C. for 18 hours. Growth was determined by the optical density measured at 660  $m\mu$ .

Although it is difficult to correlate the results of enzymatic experiments with work on whole cells, the toxicity of 5-hydroxytryptophan was examined with strain B of *E. coli* growing in a synthetic medium. As shown in Table 4, about a 50 per cent inhibition of growth was observed at the concentration of approximately  $1.5 \times 10^{-5}$  M. This inhibition was reversed by the addition of a small amount of L-tryptophan, which does not increase the final yield of the control experiment without the inhibitor (last column). 5-methyltryptophan exhibited an even stronger inhibitory effect at a comparable concentration. This may be explained by a difference in permeability or, more likely, a different site of action.

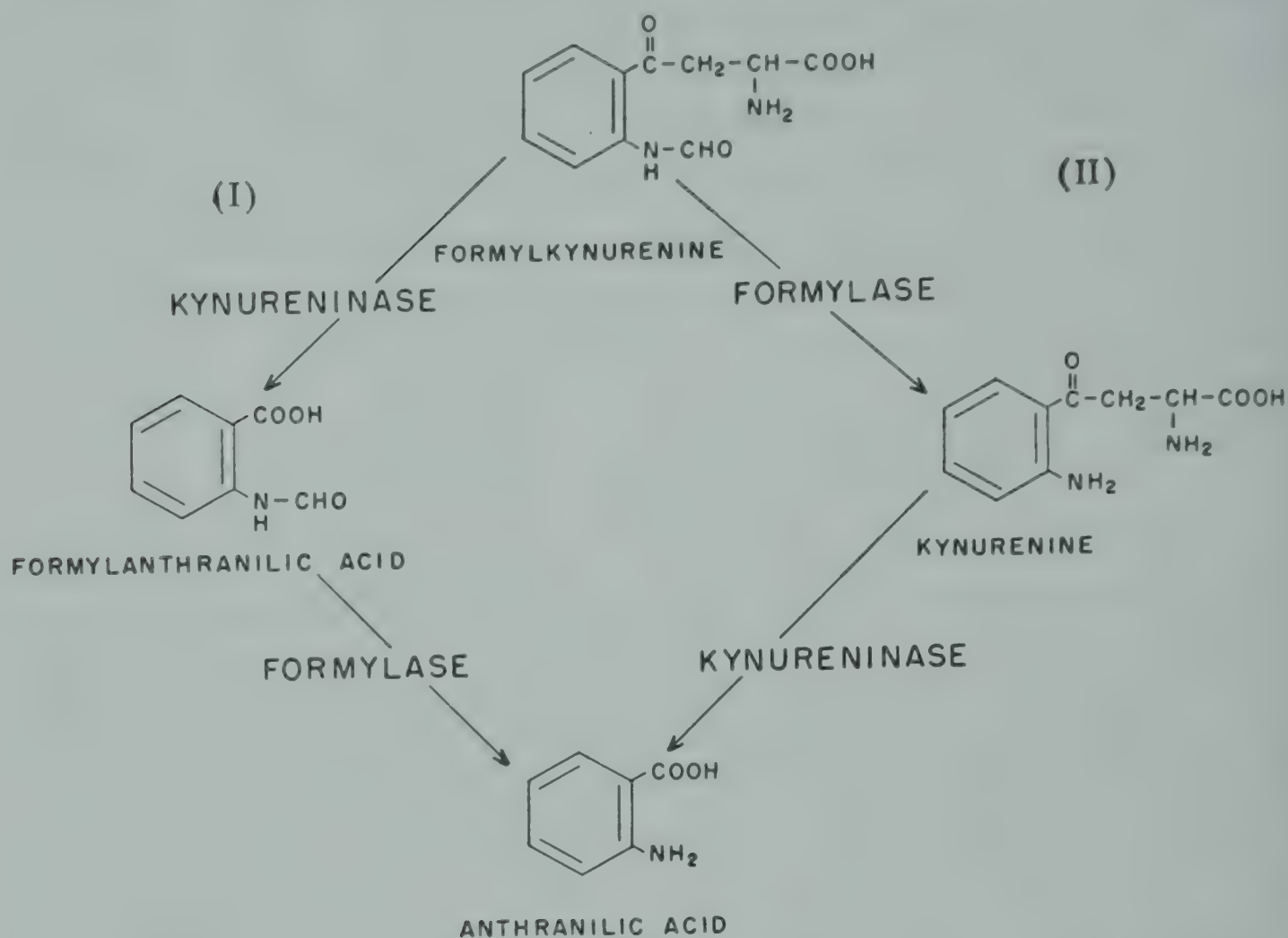
#### THE SPECIFICITY OF KYNURENINASE

It was shown by Wiss (13) that with crude rat liver kynureninase 3-hydroxykynurenine was hydrolyzed at a rate approximately twice as rapidly as kynurenine and that, besides kynurenine, a number of other compounds with the  $\alpha$ -amino- $\gamma$ -keto configuration were also metabolized. For example,  $\alpha$ -amino- $\beta$ -benzoylpropionic acid and  $\alpha$ -amino- $\gamma$ -hydroxy- $\gamma$ -phenylbutyric acid were hydrolyzed at about the same rate as kynurenine itself. More recently, the same enzyme was purified from *Neurospora* (34) and was shown to catalyze the hydrolysis of kynurenine, 3-hydroxykynurenine, and also formylkynurenine. The formation of formylanthranilic acid from formylkynurenine, as evidenced by the isolation and identification of the former compound from the incubation mixture, suggested a possibility that in vivo anthranilic acid may be produced from formylkynurenine by way of formylanthranilic acid, the latter being hydrolyzed by formylase to yield anthranilic acid (I). This pathway is an alternative to the generally held belief that formylase acts upon formylkynurenine first to yield kynurenine and formic acid, kynurenine being further split by kynureninase as shown in the scheme (II).

Crude extracts of *Pseudomonas* strain 23, grown in the presence of tryptophan, were prepared by grinding the cells with alumina, as described before (25). To 100 ml. of a crude extract were added



21 g. of ammonium sulfate, the precipitate being discarded. To the supernatant were added 10.5 g. of ammonium sulfate, this precipitate being dissolved in 40 ml. of 0.02 M. phosphate buffer, pH 7.0 (ammonium sulfate fraction). It was then acidified to pH 4.5 at



0° C. with 3% acetic acid, and the precipitate discarded. Following neutralization, the supernatant fluid was dialyzed against distilled water for 4 to 6 hours (acid-treated fraction). To 50 ml. of this preparation were added approximately 15 to 20 ml. of alumina Cy gel (18.3 mg./ml.), and the activity was eluted twice with 0.01 M. phosphate buffer, pH 7.0 (Cy fraction). To 20 ml. of Cy fraction were added 4.0 ml. of calcium phosphate gel (16.5 mg./ml.), and elution was made with 0.005 M. phosphate buffer (calcium phosphate fraction). A typical run of purification is shown in Table 5. The activity was enriched approximately 90- to 100-fold, with an overall yield of about 3 per cent.

The activity of purified bacterial kynureninase was tested by essentially the same method as described before (25), except that the reaction was always kept at neutrality, because some of the hydroxy-

TABLE 5  
PURIFICATION OF PSEUDOMONAS KYNURENINASE

	Total activity (units)	Specific activity (units per mg. protein)
Crude extract	4048	6.9
Ammonium sulfate	1726	15.7
Acid treatment	1023	42.0
Alumina C $\gamma$	410	139.0
Calcium phosphate	154	600.0

A unit of enzyme was defined as that amount which produces a density decrease of 0.100 at 360 m $\mu$  between 30 and 90 seconds after the reaction is started according to the standard assay system (see text).

lated compounds are rather unstable at alkaline reaction and undergo autooxidation and polymerization. Ten micromoles of each substrate <sup>3</sup> were incubated with 50  $\gamma$  of pyridoxal phosphate, 20 micromoles of tris buffer, pH 7.0, and 1 ml. of purified kynureninase. After the reaction was completed, the reaction mixture was deproteinized with 6% perchloric acid and the supernatant fraction, following neutralization, was streaked on Whatman No. 3 paper. Ascending chromatograms were run with two solvent systems, the one being an organic layer of butanol, water, and acetic acid mixture (5: 4: 1), and the other a propanol-water (4: 1) mixture. The compounds were visualized by a Mineralite lamp, and the spots were cut out and eluted with 0.02 M. phosphate buffer, pH 7.0. From the  $R_f$  values, the shade of fluorescence (Table 1), and the absorption spectra (Fig. 1), it was clearly established that kynureninase converts kynurenine, 3-hydroxy- and 5-hydroxykynurenine to anthranilic acid, 3-hydroxy- and 5-hydroxyanthranilic acid, respectively. The rate of each reaction was measured directly in the Beckman DU spectrophotometer by the decrease in optical density at 360, 380, and 380 m $\mu$ ,

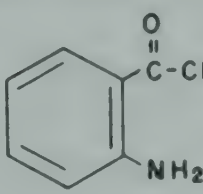
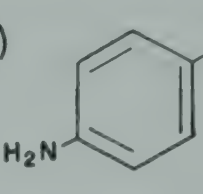
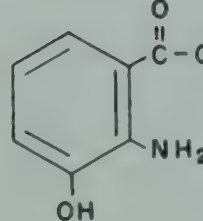
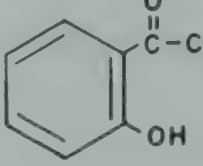
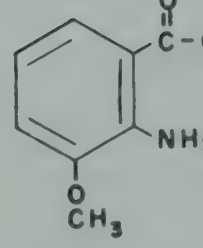
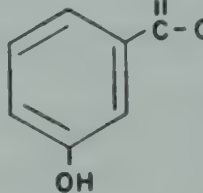
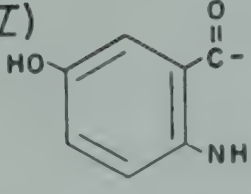
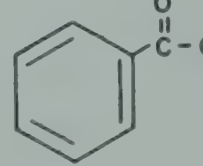
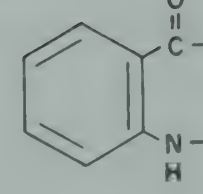
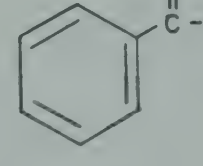
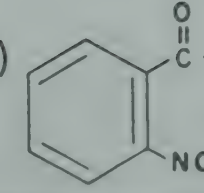
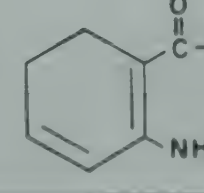
<sup>3</sup> The 3-hydroxykynurenine was a gift from Dr. A. Butenandt and Dr. V. Schiedt. 5-Hydroxykynurenine was obtained from Dr. T. Sakan and Dr. K. Makino, while 5-hydroxyanthranilic acid and xanthurenic acid were generously sent by Dr. Y. Kotake. Xanthurenic acid was also furnished by Dr. L. Musajo. L-kynurenine was synthesized by an enzymatic method previously published (29).



respectively, and was calculated from the difference of the optical density of the substrate and the product at each wave length. In contrast to the tryptophan peroxidase-oxidase system, both hydroxy-substituted kynurenines are attacked by the enzyme, but the rates are considerably less than that of unsubstituted kynurenine (Table 6).

TABLE 6

$m\mu M$ . OF SUBSTRATE DECOMPOSED PER MINUTE BY BACTERIAL KYNURENINASE

(I) 	28.0	(VII) 	0.0
(II) 	5.5	(VIII) 	3.75
(III) 	0.0	(IX) 	0.0
(IV) 	15.4	(X) 	1.67
(V) 	0.0	(XI) 	0.0
(VI) 	0.0	(XII) 	0.0

It is interesting to note that 3-hydroxykynurenine (II) is metabolized at about only one-fifth of the rate of kynurenine (1),<sup>4</sup> in view of the fact that both animal (13) and mold (34) kynureninase appear to hydrolyze the former substrate faster than the unsubstituted kynurenine.

The specificity of the purified bacterial kynureninase was investigated with various compounds related to kynurenine, which have been synthesized by Drs. T. Sakan and S. Senoh of Osaka City University. The tests were carried out as follows.

The incubation mixture (1.0 ml.) contained 0.4  $\mu$ M. of the DL-form of substrates, 50  $\mu$ M. of tris buffer, pH 7.0, 50 $\gamma$  of pyridoxal phosphate, and the purified enzyme properly diluted so as to give approximately 0.100 differences in optical density at 360 m $\mu$  per minute with the equivalent amount of L-kynurenine. When the rate of the reaction was slow, either the incubation was carried out at least one hour or else the amount of enzyme was increased several times. The rate of reaction and the identity of the product were determined spectrophotometrically in a Beckman DU spectrophotometer.

The rates of hydrolysis of various compounds are summarized in Table 6. 3-Methoxykynurenine (III) is not metabolized at all. When the aromatic amino group of kynurenine is covered with a formyl group (V) or replaced by a nitro group (VI), or moved to a para-position (VII), no appreciable enzymatic hydrolysis was observed. However, when the aromatic amino group is substituted by a hydroxy group (VIII) or totally removed (X), the resulting compounds are susceptible to the action of kynureninase, although the rates are considerably slower (approximately 13% and 6% that of the action on kynurenine, respectively). Again metahydroxybenzoyl-alanine (IX) is not metabolized at an appreciable rate. Neither *o*-aminobenzoylpropionic acid (XII) nor benzoylpropionic acid (XI) was hydrolyzed. The results seem to indicate that the aromatic amino group at the 2-position of kynurenine has a significant influence on the rate of enzymatic reaction. The relative

<sup>4</sup> The greater rate of enzymatic hydrolysis of kynurenine than 3-hydroxykynurenine by bacterial enzyme is concurrently observed by I. L. Miller and E. Adelberg (pers. commun.).



rates of decomposition of the substrates listed in Table 6 may be explained, in a qualitative sense, by the inductive and resonance effects of substituents (i. e.,  $\text{NH}_2$ ,  $\text{NO}_2$ ,  $\text{OH}$ , etc.) ortho to the amino acid side-chain and by only the inductive effect of hydroxyls meta to this side chain.<sup>5</sup> Bacterial kynureninase appears to be more specific than the same enzyme derived from the rat or *Neurospora*. In contrast to the mold enzyme, the direct formation of formyl-anthranilic acid from formylkynurenine could not be demonstrated.

### THE SPECIFICITY OF KYNURENINE TRANSAMINASE

The activity of kynurenine transaminase was tested under similar conditions, except that 20 micromoles of  $\alpha$ -ketoglutarate were added to the incubation mixture and kynurenine transaminase was used instead of kynureninase. Again the products were isolated on Whatman No. 3 paper, eluted, and a spectroscopic determination made. The identity of the products was established as the corresponding kynurenic acid derivatives (see Fig. 1). The transaminase appears to be even more non-specific than the kynureninase, since the rate of formation of xanthurenic acid, as measured by the increase of absorption at  $340\text{ m}\mu$ , is approximately 77 per cent that of kynurenic acid formation from kynurenine, which in turn was determined by the increase of optical density at  $330\text{ m}\mu$ . With 5-hydroxykynurenine as a substrate, a new compound, presumably 6-hydroxykynurenic acid, was produced. Assuming an extinction coefficient at  $350\text{ m}\mu$  (maximum at pH 7.0) as  $8,000\text{ cm}^2/\text{mole}$ , the rate of formation of 6-hydroxykynurenic acid appears to be about 36 per cent of that of kynurenic acid.

The absorption spectra of enzymatically formed 6-hydroxykynurenic acid at 3 different pH's and the paper chromatographic data agree completely with the results obtained with chemically synthesized 6-hydroxykynurenic acid<sup>6</sup> (see Fig. 3, Table 1). It is to be noted that the 6-position in the quinoline structure is para to the heterocyclic nitrogen atom, and that hydroxylation in position 6 has

<sup>5</sup> The author is indebted to Dr. P. Lipkin for suggesting this interpretation.

<sup>6</sup> Kindly furnished by Dr. K. Makino, Kumamoto Medical College, Japan.

been shown to occur with quinoline (35) as well as 2- and 4-quinolones (36). It is, therefore, quite conceivable that kynurenic acid may be directly hydroxylated *in vivo* to form 6-hydroxykynurenic acid, although the latter compound has not been isolated from natural sources.

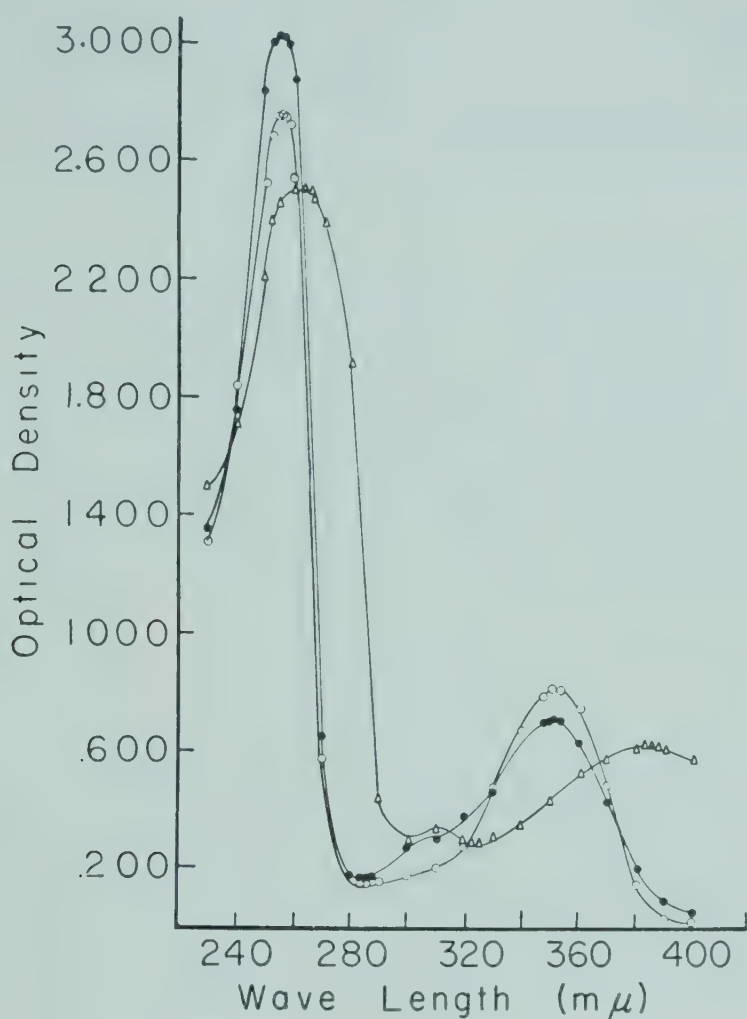


FIG. 3. Spectra of a product of the transaminase reaction from 5-hydroxykynurenine and 6-hydroxykynurenic acid.

Concentration is approximately  $10^{-4}$  M.

□: pH 9.8. }  
 ○: pH 6.9. } (Enzymatic product)  
 ●: pH 2.8. }

Continuous line (synthetic material).

### SUMMARY

The results presented in this discussion demonstrate that the tryptophan peroxidase-oxidase system requires strict specificity on the benzene part of the tryptophan molecule and indicate that 5- and 7-hydroxytryptophan are not converted to the corresponding hydroxy-substituted kynurenine derivatives at an appreciable rate.



5-hydroxytryptophan and also 7-hydroxytryptophan, to a lesser extent, competitively inhibit the enzymatic oxidation of L-tryptophan.

Kynureninase is less specific and converts kynurenine and 3- and 5-hydroxykynurenine to the corresponding anthranilic acid derivatives, the ratio of the rate of reaction being approximately 10: 2: 5. However, formylkynurenine is not metabolized to an appreciable extent. There is some evidence that the aromatic amino group, in addition to the aliphatic amino group, is essential for the enzyme action.

Kynurenine transaminase is much less specific and converts kynurenine, 3- and 5-hydroxykynurenine to kynurenic acid, 8- and 6-hydroxykynurenic acid, the ratio of the rate of reaction being approximately 10: 8: 4. A new compound, 6-hydroxykynurenic acid, was enzymatically synthesized from 5-hydroxykynurenine.

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# TRYPTOPHAN AND NIACIN SYNTHESIS IN VARIOUS ORGANISMS

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THE AMINO ACID tryptophan and the vitamin niacin are essential components of living matter. Various organisms are dependent for survival upon an external supply of these two compounds, while other organisms can synthesize one or both. In those organisms which are capable of synthesizing tryptophan, niacin, or both, it is of interest from an evolutionary and comparative biochemical standpoint to examine whether or not similar biosynthetic pathways of tryptophan and niacin synthesis are employed. In this discussion I will consider the information available at present concerning the pathways of tryptophan and niacin synthesis in various organisms.

## TRYPTOPHAN SYNTHESIS

A general scheme of tryptophan synthesis in microorganisms involving anthranilic acid and indole as intermediary compounds

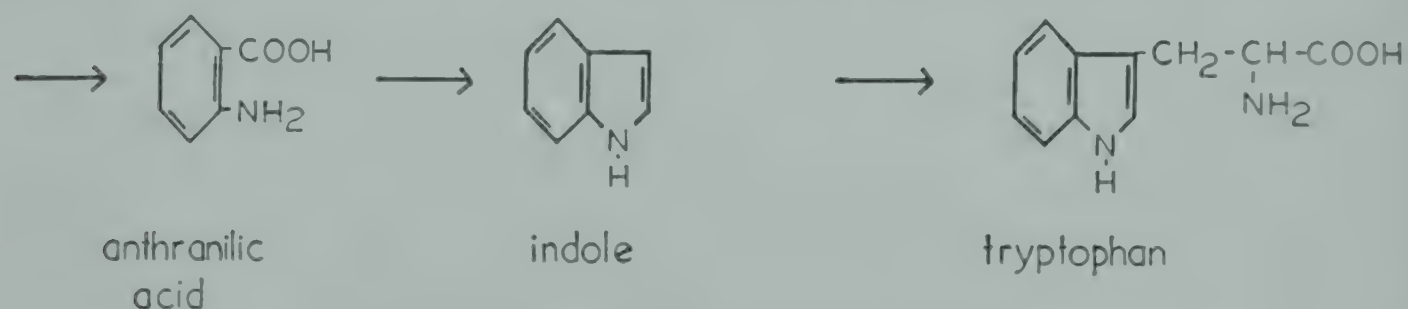


FIG. 1. Scheme of tryptophan synthesis.

(Fig. 1) was based on the initial observations of Fildes (1) and Snell (2). The nature of the precursors of anthranilic acid and of all the aromatic amino acids has been discussed previously in this

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symposium. I will consider here only those steps between anthranilic acid and tryptophan.

### *The Conversion of Indole to Tryptophan.*

Tryptophan auxotrophs of *Neurospora* (3) and of *Escherichia coli* are known whose tryptophan requirement cannot be satisfied by indole. Such strains characteristically accumulate indole and anthranilic acid (4, 5) in their culture filtrates, as might be expected from the position of their biochemical block. These strains, when examined enzymatically, are found to lack tryptophan synthetase (3, 4, 5), the enzyme which catalyzes the coupling of indole and L-serine in wild-type strains of *Neurospora* (6, 7, 8) and *E. coli* (9). The fact that tryptophan synthetase is not formed by mutant strains blocked in the conversion of indole to tryptophan indicates that the coupling of indole and serine is the principal reaction by which tryptophan is formed in *Neurospora* and *E. coli*. This conclusion is supported by the observation that uniformly labeled indole is converted to tryptophan, without dilution, by a tryptophan auxotroph of *E. coli* (9).

### *The Conversion of Anthranilic Acid to Indole.*

In addition to the class of tryptophan auxotrophs mentioned above which cannot use indole for growth, in both *Neurospora* (10) and *E. coli* other tryptophan auxotrophs are known which respond to either indole or tryptophan but not to anthranilic acid. In *Neurospora* two distinct genetic types have been recognized in this second class of tryptophan auxotrophs. Both genetic types accumulate anthranilic acid in their culture filtrates. Anthranilic acid is also accumulated by tryptophan auxotrophs of *E. coli* which are blocked in the conversion of anthranilic acid to indole. The existence of two distinct genetic types in *Neurospora* blocked in the conversion of anthranilic acid to indole has been taken as suggestive evidence that at least two distinct steps are involved in this conversion. It is known from isotope experiments performed with *Neurospora* (11) that the carboxyl group of anthranilic acid is lost during conversion to indole. In *E. coli* it has been possible to show, using washed cell suspensions, that during the conversion of unlabeled anthranilic acid to indole in



the presence of uniformly labeled glucose, two carbon atoms of the indole formed are derived from the labeled glucose (5). This suggests that in *E. coli*, as in *Neurospora*, the carboxyl group of anthranilic acid is lost during conversion to indole.

In the same *E. coli* system, it has been possible to show that the pyrrole ring of indole is formed through the carbon atom of the benzene ring to which the carboxyl group of anthranilic acid was

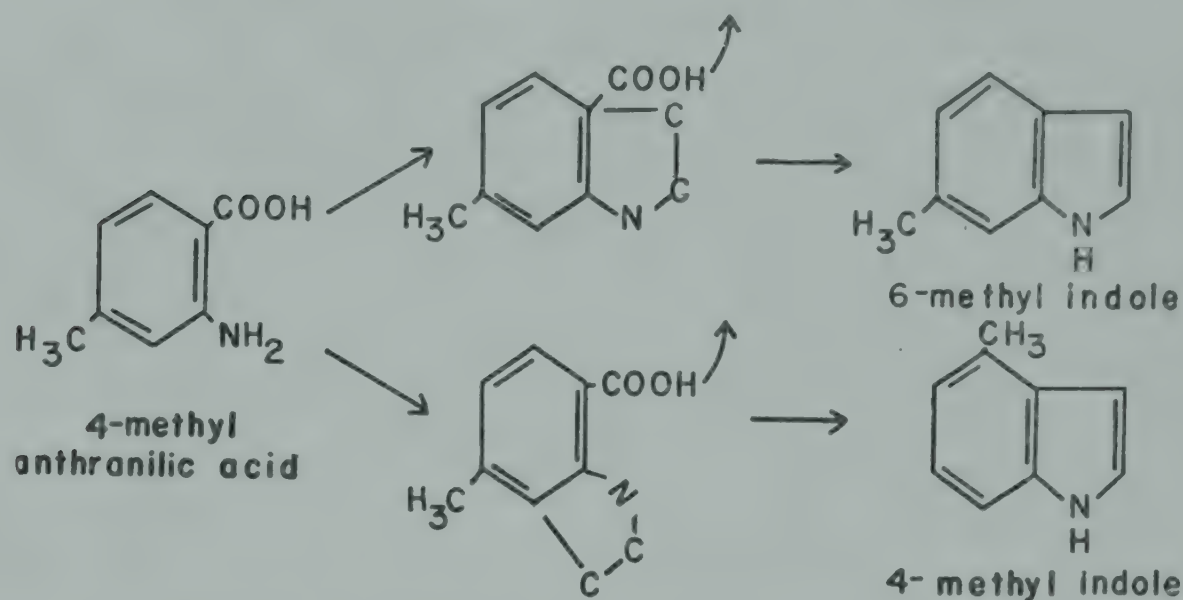


FIG. 2. The action of *E. coli* suspensions on 4-methylanthranilic acid. 4-methylindole would be formed if the pyrrole ring was closed through position 3 of the benzene ring, 6-methylindole would be formed if the pyrrole ring was closed through position 1.

previously attached (5). *E. coli* suspensions, in addition to converting anthranilic acid to indole, also convert 4-methylanthranilic acid to an indole derivative. If ring closure in 4-methylanthranilic acid occurred through position 1 of the benzene ring, the product of the reaction should be 6-methylindole (Fig. 2). If, however, ring closure occurred through position 3 of the benzene ring, then 4-methylanthranilic acid should give rise to 4-methylindole and not to 6-methylindole. An experiment of this nature was carried out with 4-methylanthranilic acid, and the indole derivative formed was isolated and identified as 6-methylindole. Thus, ring closure in the formation of indole occurs through the carbon atom to which the carboxyl group of anthranilic acid was attached. This finding also suggests that decarboxylation is not the first step in the conversion of anthranilic acid to indole. If decarboxylation were the first step, then a mixture of 4-methylindole and 6-methylindole might have

been expected in the previous experiment. In agreement with the conclusion that decarboxylation probably is not the first step is the observation that a cell suspension of *E. coli* which readily converts anthranilic acid to indole will not act on aniline (5).

*Is Anthranilic Acid on the Pathway of Tryptophan Synthesis?*

The facts that anthranilic acid supports the growth of some tryptophan auxotrophs and is accumulated by others would ordinarily be considered fairly conclusive evidence that anthranilic acid is an intermediate on the pathway of tryptophan synthesis. Haskins and Mitchell (12), however, have shown that anthranilic acid is formed

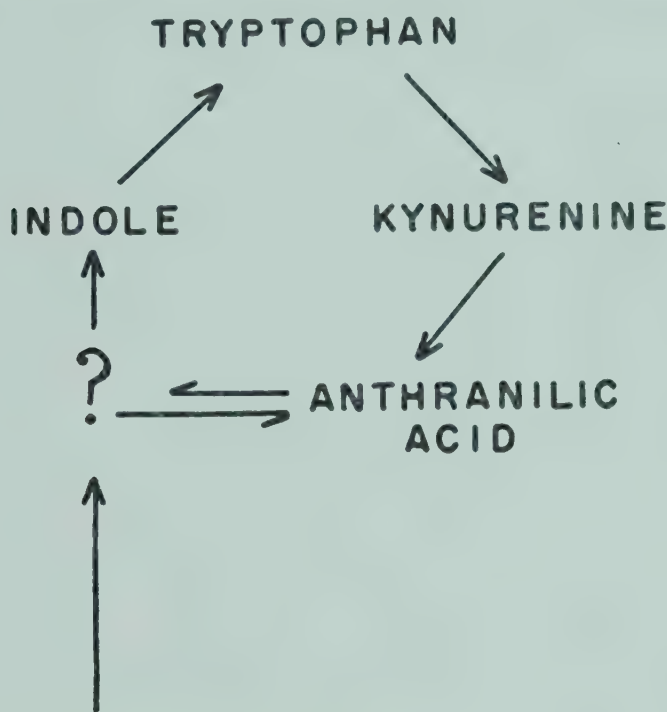


FIG. 3. The "tryptophan cycle" of Haskins and Mitchell.

in two ways by *Neurospora* and have made the interesting proposal that anthranilic acid is not on the path of tryptophan synthesis but is readily converted to and readily formed from a "true" intermediate. The two ways by which anthranilic acid can be formed in *Neurospora* and the proposed mechanism (12) by which it is converted to tryptophan are illustrated in Fig. 3.

The same situation does not appear to obtain in *E. coli* (9). Thus, although the "tryptophan cycle" accounts for the ability of kynurenine to support the growth of anthranilic-acid-responding tryptophan auxotrophs of *Neurospora*, similar auxotrophs of *E. coli* do not respond to kynurenine. Furthermore we have been unable



to detect the formation of anthranilic acid from kynurenine<sup>1,2</sup> or of kynurenine from tryptophan in experiments with *E. coli*.

The combined observations in *Neurospora* and *E. coli* strongly suggest that anthranilic acid is an intermediate in tryptophan synthesis in these two organisms. The proposal of Haskins and Mitchell, however, cannot be ruled out by any of the existing data. It appears that final evaluation of this situation must await a better understanding of the reactions leading to the formation of indole.

*Tryptophan Synthesis in Bacillus subtilis and Lactobacillus arabinosus.*

Tryptophan auxotrophs of *B. subtilis* fall into the same categories as the tryptophan auxotrophs of *E. coli* and *Neurospora*. Thus, some tryptophan auxotrophs do not respond to anthranilic acid or indole, some respond to indole as well as tryptophan, and some respond to anthranilic acid, indole, or tryptophan. Although this picture seems to be similar in all respects to that present in *E. coli* and *Neurospora*, there is one discrepancy. Those *B. subtilis* mutants which are blocked in the conversion of indole to tryptophan do not accumulate indole or anthranilic acid in their culture filtrates (13). It will be remembered that this property is characteristic of the *Neurospora* and *E. coli* mutants blocked in this reaction. Whether or not this difference is indicative of a different path of tryptophan synthesis in *B. subtilis* cannot be stated at present.

The utilization of anthranilic acid and indole as precursors of tryptophan in *Lactobacillus arabinosus* has been examined by Rhuland and Bard (14), who have concluded from their data that the pathway of tryptophan synthesis in this organism is considerably different from the pathway which is operative in *Neurospora*. These authors suggest that indole is not a product of anthranilic acid utilization and that indole is not converted to tryptophan by coupling with serine. If these conclusions can be supported by more direct evidence it would be the first clear case of a second pathway of tryptophan synthesis.

<sup>1</sup> *E. coli* does, however, form kynurenic acid from kynurenine.

<sup>2</sup> Cell-free extracts of *E. coli* and *B. subtilis* are also incapable of converting kynurenine to anthranilic acid (Dr. W. B. Jakoby, pers. commun.).

# NIACIN SYNTHESIS

Investigations with mammals and with *Neurospora* (for reviews, see 15 and 16) have led to the scheme of niacin synthesis shown in Fig. 4. There do not appear to be any major qualitative differences

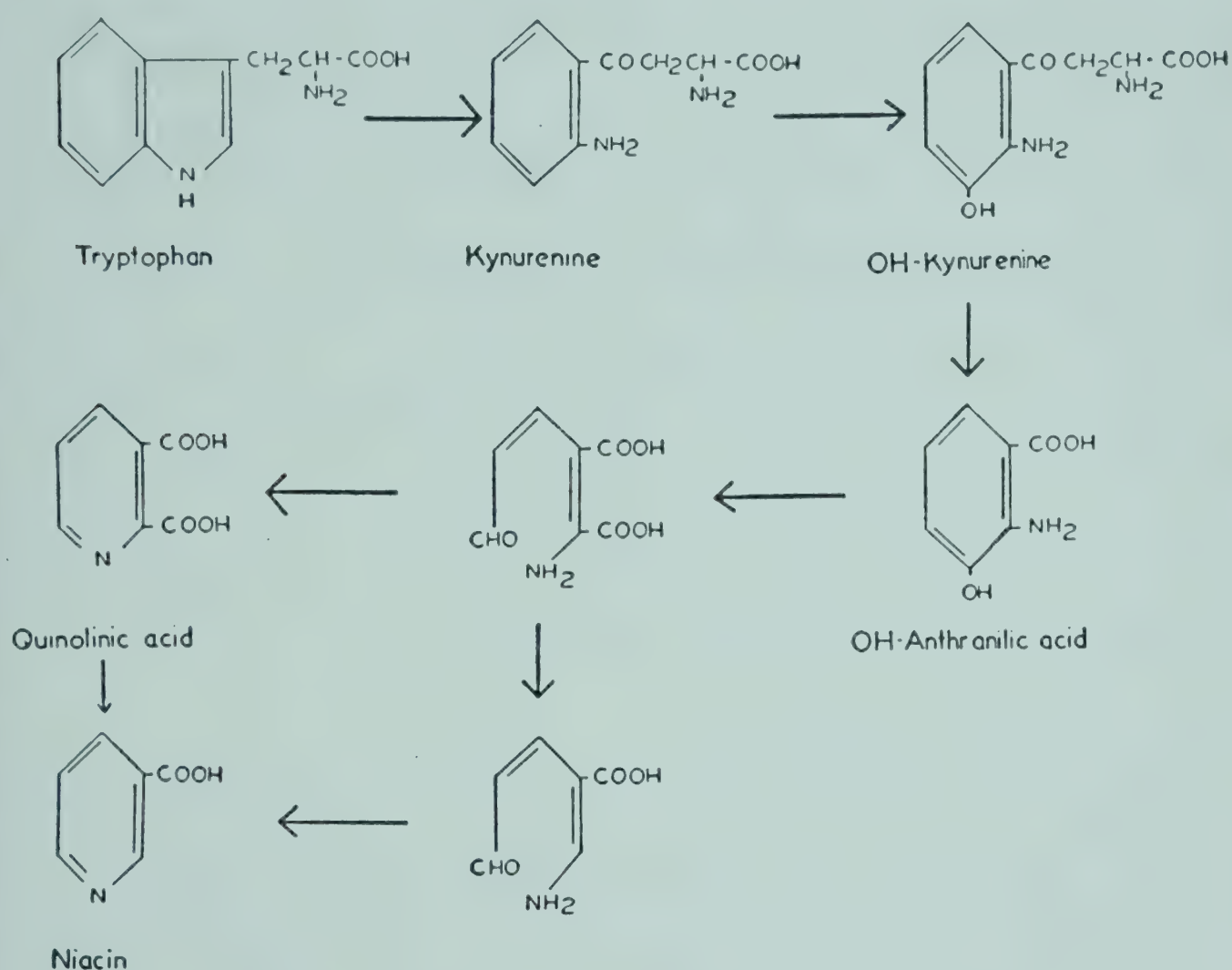


FIG. 4. Scheme of niacin synthesis from tryptophan.

between *Neurospora* and rats in regard to this scheme. The only differences which have been observed seem to be of a quantitative nature.

## Individual Steps in Niacin Synthesis.

Conversion of tryptophan to kynurenine has been demonstrated enzymatically with mammalian preparations and has been shown to involve N-formylkynurenine as an intermediary compound (17, 18). The same reactions would appear to be involved in kynurenine formation in *Neurospora*; however, only the step from N-formylkynurenine to kynurenine has been demonstrated (19). The enzyme



catalyzing the conversion of kynurenine to 3-hydroxykynurenine has not been obtained from either *Neurospora* or mammalian tissues. Conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid is catalyzed by kynureninase (20, 21), an enzyme which also converts kynurenine to anthranilic acid. Intermediary steps in the conversion of 3-hydroxyanthranilic acid to niacin, involving the conversion of a benzene ring to pyridine ring, remain obscure. Formation of quinolinic acid, either as a direct intermediate or as a by-product, is the only clue available at present as to the mechanism of this reaction (22, 23, 24). 3-Hydroxyanthranilic acid conversion to quinolinic acid is catalyzed by rat liver preparations (25). However, here again, the details of the reaction are unknown, although the existence of an intermediary compound has been demonstrated (26).

Direct support for the conversion of tryptophan to niacin has been obtained in isotope experiments performed both with mammals (27) and *Neurospora* (28). The results of the isotope experiments performed with *Neurospora* indicate that in this organism niacin synthesis proceeds solely from tryptophan.

#### *Niacin Synthesis in Bacteria.*

Comparatively little is known about niacin synthesis in bacteria. Evidence for the participation of tryptophan as a precursor of niacin has been presented for the bacterium *Xanthomonas pruni* (29). This organism has a niacin requirement which can be satisfied by tryptophan and other compounds which are intermediates in the conversion of tryptophan to niacin in *Neurospora*. In *E. coli* the pathway of niacin synthesis has been assumed by Marnay (30) to be the same as in *Neurospora* on the basis of inhibition studies. Other workers, however, have suggested that tryptophan does not serve as a niacin precursor in *E. coli* but functions catalytically in its formation (31).

During the past year I have had occasion to examine the existence of a tryptophan-niacin relationship in *E. coli* and also in *B. subtilis* (9). The results of this investigation in fact suggested that such a relationship does not exist in these bacteria. Thus, niacin auxotrophs of both organisms do not respond to any of the compounds which are effective niacin substitutes for niacin auxotrophs of *Neuro-*

*spora*. Furthermore, neither of the strains we have examined appear to be capable of converting kynurenine to anthranilic acid. Since the enzyme catalyzing this reaction, kynureninase, is required for the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid, we may assume that the latter reaction, an essential step in niacin synthesis in mammals and *Neurospora*, is not involved in niacin synthesis in *E. coli* or *B. subtilis*.

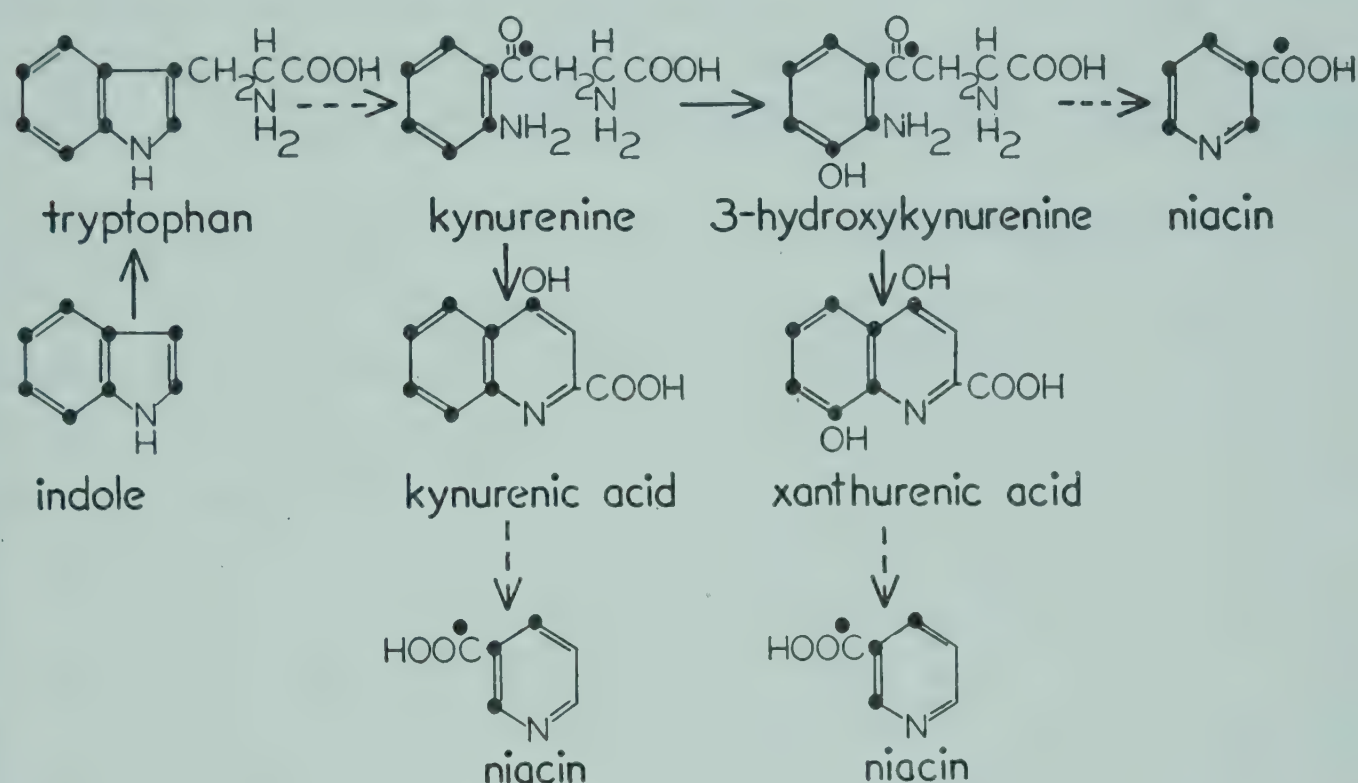


FIG. 5. Possible pathways of niacin formation from indole or tryptophan.

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● indicates carbon atoms which would be expected to be labeled.

To obtain more direct evidence bearing on the question of whether or not tryptophan serves as a precursor of niacin in *E. coli* or *B. subtilis*, isotope experiments were carried out with tryptophan auxotrophs of these organisms (9). The strains employed were blocked in the conversion of anthranilic acid to indole and could use either indole or tryptophan for growth. Since they required exogenous indole or tryptophan, the niacin formed during growth, if it were derived from tryptophan, could only have come from exogenous indole or tryptophan. Thus, by labeling the indole or tryptophan supplied it was possible to determine whether or not niacin was formed from these compounds. In Fig. 5 I have indicated the



expected labeling in niacin if niacin synthesis proceeded by any of the three tryptophan pathways illustrated. The results of the isotope experiments showed conclusively that neither indole nor tryptophan serves as a major precursor of niacin in the *E. coli* or *B. subtilis* strains examined. Thus, it was possible to eliminate all three of the pathways of niacin synthesis illustrated in Fig. 5. The existence of a minor pathway of niacin synthesis from tryptophan is not excluded by the isotope data. In view of the results obtained, however, a pathway from tryptophan, if present in the two bacterial species, could provide at most about five per cent of the niacin which is formed.

As yet no evidence is available as to the nature of the precursors or the pathway by which niacin is synthesized in *E. coli* and *B. subtilis*. Attempts to stimulate niacin production by the addition of various amino acids, either alone or in combination, have been unsuccessful. Furthermore, several of the intermediates between tryptophan and niacin in *Neurospora* have been tested and found ineffective in boosting niacin accumulation during growth. Ornithine was also tested, since it had previously been shown that in some strains of *E. coli* (32) it stimulates niacin production. In neither of the species examined by us did it stimulate niacin formation.

### CONCLUSIONS

From an evolutionary and biochemical standpoint it is interesting to note that *Neurospora*, *E. coli*, and *B. subtilis* appear to employ similar mechanisms for tryptophan synthesis. There is suggestive evidence that *L. arabinosus* employs a different pathway; however, more direct evidence with this organism would be desirable.

Although these organisms utilize the same pathway of tryptophan synthesis, *Neurospora* employs tryptophan as a precursor of niacin, whereas *E. coli* and *B. subtilis* do not. In view of this major difference with respect to niacin synthesis, it becomes even more interesting and perhaps surprising that the path of niacin synthesis from tryptophan appears to be identical in the mold *Neurospora* and in some mammals.

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# GENETIC CONTROL OF TRYPTOPHAN METABOLISM IN DROSOPHILA \*

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FOR THE LAST several years the authors (1-4, 8) have been much interested in a strain of *Drosophila melanogaster* in which two sorts of abnormal growth are produced in almost all of the treated individuals when embryos are x-rayed. Just before pupation, each of the larvae develops one or more melanotic tumors; and after metamorphosis each adult exhibits deranged eyes, in most instances with a growth protruding through the center of the eye and surmounted by small bristles (1). Genetic analysis, carried out by Mr. Palmer Rogers and myself, has demonstrated that this strain of *Drosophila* carries two distinct mutant genes, one responsible for the melanotic tumors and located in chromosome 2; the other responsible for the erupt eyes, and located in chromosome 3. Normally both mutant effects are completely suppressed by the simultaneous presence of two specific suppressor genes, a suppressor of erupt in chromosome 2, and a suppressor of the tumor gene, probably in chromosome 3. The effect of the x-rays upon embryos or larvae of this strain is, then, to block in some way the action of the suppressor genes, so that the mutants, no longer inhibited, modify the phenotype in their characteristic ways.

The effective action of the x-rays depends upon the oxygen concentration of the atmosphere at the time of irradiation (3, 8). It is almost completely nullified in the absence of oxygen, and is enhanced when the irradiation takes place in pure O<sub>2</sub>. When the embryos are exposed to solutions of hydrogen peroxide for a brief period, both erupt eyes and melanotic tumors are significantly increased in frequency (6). When larvae, prior to being irradiated

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with x-rays 24 hrs. after hatching, are fed on a complete medium supplemented with 0.1% of L-cysteine, the incidence of tumors is reduced from 78.2% to 46.1%; and when cysteine is fed only *after* the treatment with x-rays, the incidence is reduced to 32.4% (7). When cysteine is fed both before and after irradiation, only 13.0% of the larvae develop tumors. Cysteine fed in this manner without irradiation totally lacks effect. Cysteine similarly counteracts the effect of x-rays in blocking the suppressor of erupt eyes (7). It reduces the incidence of erupt eyes from 90.0% to 13.9%, when fed both before and after an irradiation at 24 hrs. of larval age. In this case, however, feeding with cysteine before irradiation has a greater effect than feeding with cysteine after the x-ray treatment, although the latter also significantly reduces the x-ray effect.

These results with x-rays, oxygen, peroxide, and cysteine are described at the present time because the action of L-tryptophan upon the two suppressor gene effects is strikingly similar to that of x-rays. When larvae are reared upon a complete medium supplemented with 0.5% of L-tryptophan, about three times the amount normally present, the tumor incidence rises from 4.4% to 63.2%, and the incidence of erupt eyes from 9.2% to 51.6%. Further supplementation with 0.1% of cysteine reduces the incidence of tumors to 24.2%, and the incidence of erupt eyes to 7.0%. Exposure of the embryos to oxygen for 10 mins. markedly enhances the effect of subsequent feeding of the larvae upon tryptophan.

The effects of other aromatic amino acids added as supplements to a complete medium, and in a concentration similar to that used for tryptophan, are much less striking. L-Phenylalanine has no effect upon the suppressor of erupt eyes, and a barely significant effect upon the suppressor of the tumor gene. L-Tyrosine possesses a stronger effect; it increases the erupt eye incidence to 20.2% and the tumor incidence to 24.5%. Thus the abolition of the suppressor actions of the two genes is not entirely specific to tryptophan. Yet the effect of the latter is so much greater that these results suggest that the two forms of abnormal growth are directly related to tryptophan metabolism. From the evidence that peroxides may be involved,



interest naturally centers on the step whereby tryptophan is oxidized by a peroxidase to formylkynurenine. Work just being completed indicates that kynurenine itself (kindly supplied by Dr. David Bonner) is effective, at least in the counteraction of the tumor suppressor.

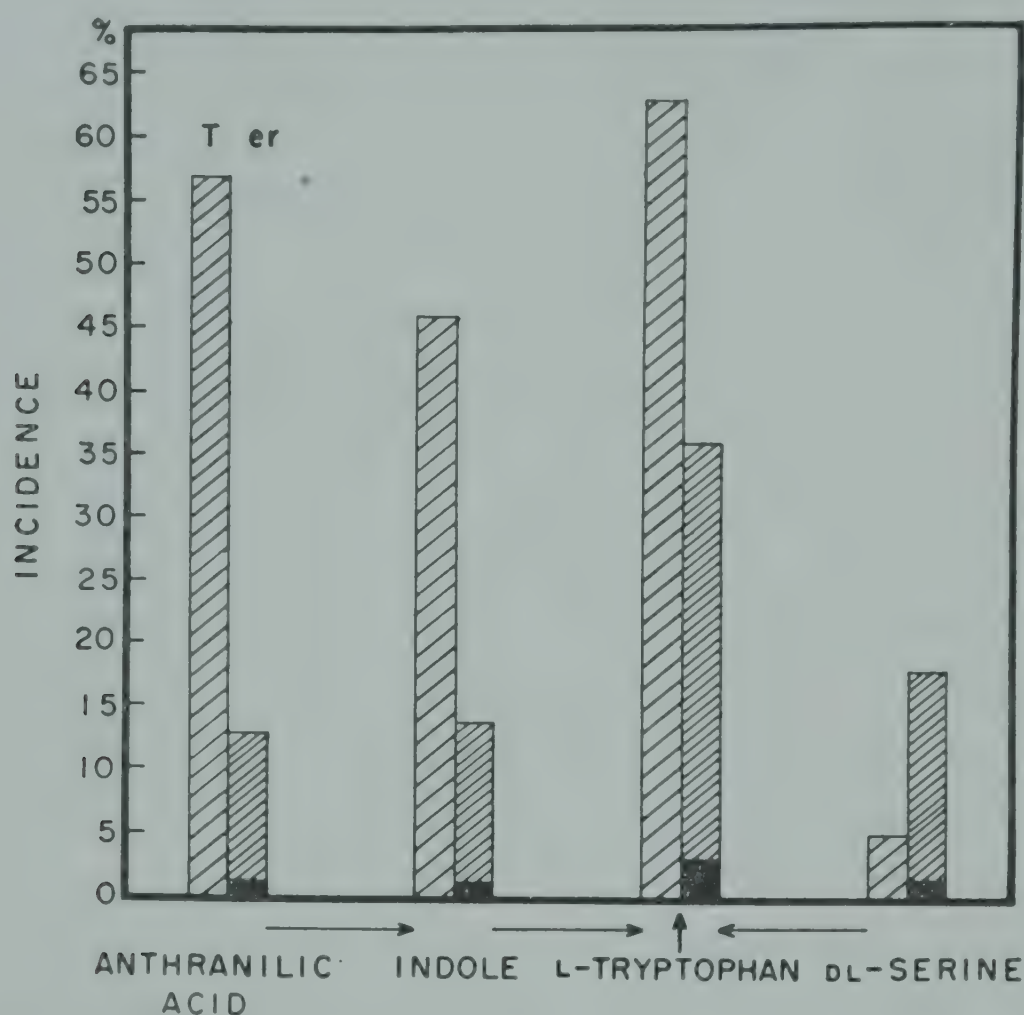


FIG. 1. The incidence of melanotic tumors and erupt eyes produced in the suppressor-erupt stock by supplementary feeding with anthranilic acid (5%), indole (0.1%), L-tryptophan (5%), or DL-serine (5%), mixed dry with brewers' yeast, and moistened. For each substance, the column to the left (T) shows the percentage of third instar larvae with tumors, and the column to the right (er) shows the percentage of imagoes with erupt eyes. The lower, darker portion of the right-hand column represents the frequency of flies classified as extreme erupt; the upper part of this column represents the frequency of flies with a weak manifestation of erupt.

The known pathway of tryptophan metabolism in *Drosophila* leads from tryptophan through formylkynurenine to kynurenine, and then to 3-hydroxykynurenine, and thence to the brown eye pigments (5). This system is duplicated in *Bombyx*, the silkworm, and in *Ephestia*, the meal moth. There are a few branch paths known in the silkworm (5); e. g., kynurenine may go to anthranilic acid and alanine, and thence to anthranilylglycine; and 3-hydroxykynurenine goes to 3-

hydroxyanthranilic acid and 3-hydroxyanthranilylglycine and perhaps other products, although not to nicotinic acid. If *Drosophila* possesses a kynureninase, anthranilic acid and alanine might possibly lie on the tryptophan path leading to melanotic tumors or erupt eyes; or if it possesses a transaminase, kynurenic acid might play a role. The effects of anthranilic acid and L-alanine have been tested

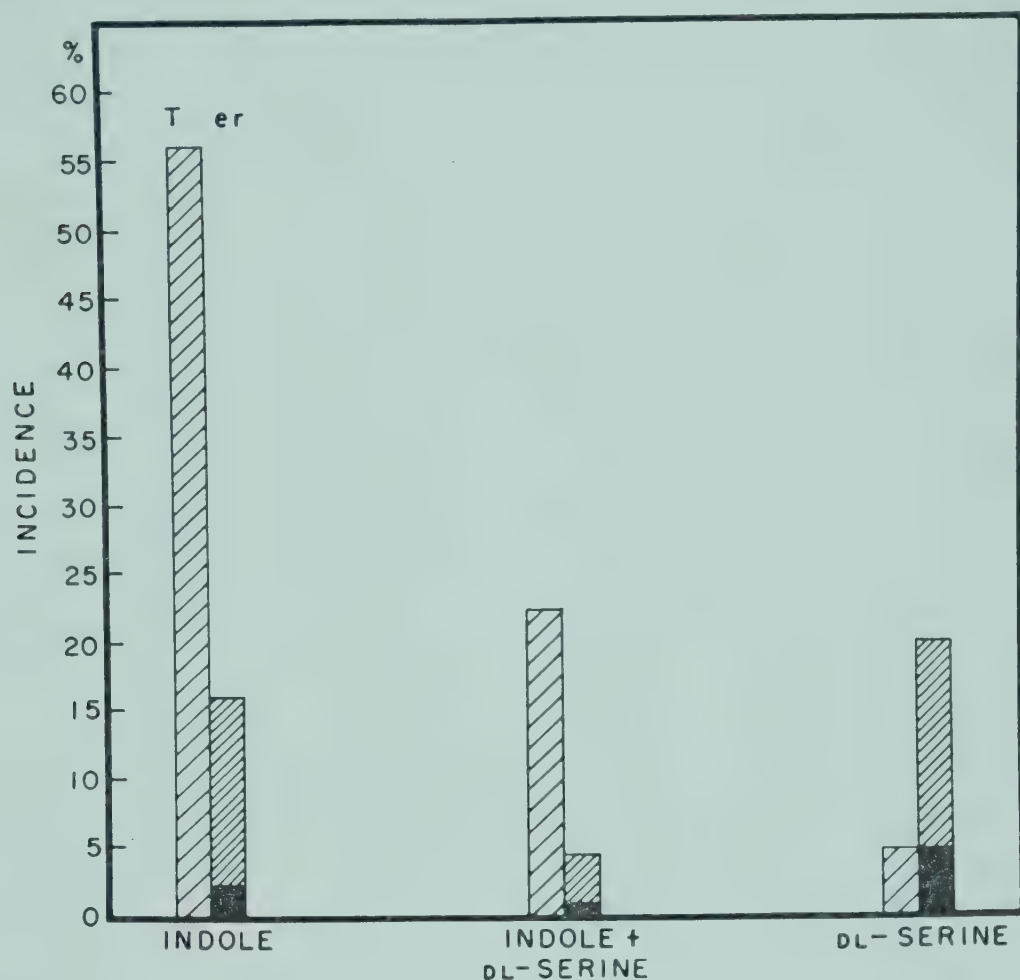


FIG. 2. The incidence of melanotic tumors and erupt eyes produced in the suppressor-erupt stock by supplementary feeding with indole (0.01%), DL-serine (0.5%), or indole + DL-serine (same concentrations), in medium of 5% brewers' yeast and 1.5% agar in water. For each substance, the column to the left (*T*) shows the percentage of third instar larvae with tumors, and the column to the right (*er*) shows the percentage of imagoes with erupt eyes. The lower, darker portion of the right-hand column represents the frequency of flies classified as extreme erupt; the upper part of this column represents the frequency of flies with a weak manifestation of erupt.

separately on the action of the suppressor genes (9). The former is very toxic, but produced an incidence of melanotic tumors equal to that produced by tryptophan, whereas its effect in increasing the incidence of erupt eyes was slight. L-Alanine (0.5%) produced 17.4% of individuals with tumors, 20.0% with erupt eyes. Its effect is thus about equal to that of tyrosine. The precise interpretation of these results remains unclear at present.



Inasmuch as *Neurospora* is known to couple indole and serine to produce tryptophan (10), it seemed of interest to test the effects of these two compounds. The results, together with the effects of anthranilic acid, which in *Neurospora* is a precursor of indole, are shown in Fig. 1 (4, 9). Of the two components of the tryptophan molecule, indole affects mainly the incidence of tumors, and serine affects only the occurrence of erupt eyes. Fig. 2 shows the effect of supplementing the complete medium with both indole and DL-serine. Although this combination retained an effect upon the incidence of melanotic tumors, it is clearly not equivalent to the effect of tryptophan, nor even to that of indole alone; and there was no increase at all in the incidence of erupt eyes, over and above that of the controls. Whether these effects are due to competition between tryptophan and indole and between tryptophan and serine in distinct paths leading to the two types of abnormal growth; or whether tryptophan can separately undergo cleavage to indole, pyruvic acid, and ammonia, as in *E. coli*, or via kynurenine to anthranilic acid and alanine (or serine) cannot be said now—but clearly there are many possibilities worthy of assay.

In short, it appears at present that two distinct suppressor genes which inhibit different types of abnormal growth in *Drosophila* alike produce their effects by raising the threshold of response to tryptophan or some oxidized derivative above that level of response which characterizes the respective mutant types. Any mechanism that increases the supply of tryptophan or its active derivative tends to overwhelm the action of the suppressor genes and thus to result in the characteristic effects of the erupt-eye and melanotic tumor mutant genes.

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# THE 5-HYDROXYINDOLE PATHWAY OF TRYPTOPHAN METABOLISM

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THE ISOLATION and identification of serotonin, the vasoconstrictor substance in blood, as 5-hydroxytryptamine (5HTA) by Rapport and Page (5) introduced a new physiologically occurring indole. This laboratory has been investigating the biosynthesis and metabolism of this compound. The dietary precursor of 5HTA has been found to be tryptophan. This was demonstrated in rabbits and toads

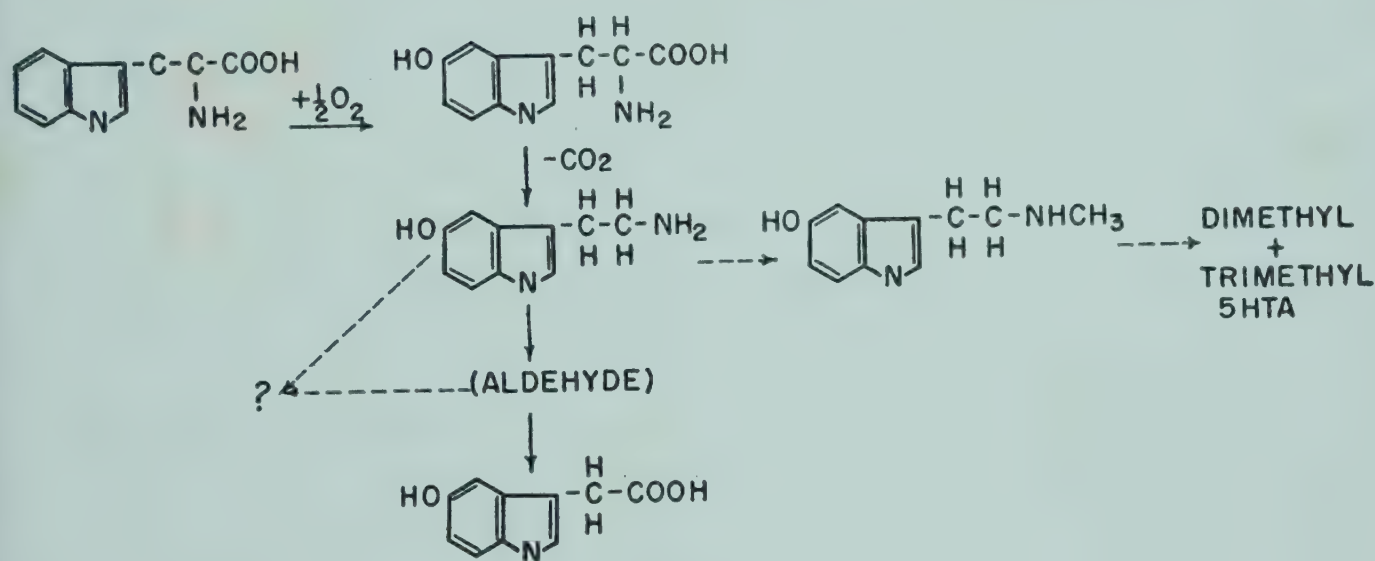


FIG. 1.

by isolating radioactive 5HTA after feeding  $C^{14}$ -tryptophan. 5HTA isolated after  $C^{14}$ -tyrosine contained no radioactivity. Thus, 5HTA appears to be an intermediate in the following route of metabolism of tryptophan (Fig. 1).

The conversion of tryptophan to 5HTA involves decarboxylation and then hydroxylation of an aromatic ring. Although tryptamine suggested itself as a likely intermediate, no evidence for this com-



pound nor for its enzymatic formation could be demonstrated in a large number of tissues in rats, guinea pigs, rabbits, or toads. Slices and homogenates of the tissues were incubated anaerobically with 10-20  $\mu$ M. of L-tryptophan for several hours. Tryptamine was not metabolized at all when incubated anaerobically with any of these tissues. As little as 0.02 micromoles of tryptamine could have been detected in these experiments.

Previous reports by Werle et al. (8) indicating the ability of mammalian tissue to convert tryptophan to a pressor substance were probably correct. However, the pressor substance which they thought to be tryptamine was most likely 5-hydroxytryptamine.

Evidence for the existence of 5-hydroxytryptophan was obtained from paper chromatograms of toad venom. The amounts occurring in the venom are small, and the amino acid is relatively labile. When 10 microcuries of  $C^{14}$ -tryptophan were administered to a toad, the 5-hydroxytryptophan isolated from the venom with the aid of carrier contained appreciable radioactivity. Radioactivity remained even after conversion to a *p*-iodophenylsulfonyl (PIPSYL) derivative. Further the specific activity of the PIPSYL derivative remained constant following recrystallization (Table 1).

TABLE 1  
CONVERSION OF  $C^{14}$ -TRYPTOPHAN TO 5-OH-TRYPTOPHAN  
BY THE TOAD VENOM GLAND IN VIVO

Crystallization	c.p.m./mg.	Total 5-OH-tryptophan Activity
		c.p.m.
2	116	694
4	118	707

1  $\mu$ C. of 2- $C^{14}$ -DL-tryptophan administered daily for 7 days; gland excised, extracted, and chromatographed on paper. 5-OH-tryptophan area eluted, mixed with 6 mg. of carrier, and treated with *p*-iodophenylsulfonyl chloride to make the PIPSYL derivative, which was then recrystallized to constant specific activity.

Experiments in vitro indicate that guinea pig and rat liver slices can form 5-hydroxytryptophan from tryptophan. These experiments were carried out with  $C^{14}$ -tryptophan; non-isotopic 5-hydroxytryptophan was used as a trap.

By far the most convincing evidence for the existence of this new amino acid is the demonstration of a specific 5-hydroxytryptophan decarboxylase in many animal tissues. The enzyme from hog and guinea pig kidneys has been isolated and purified approximately 25-fold. Its properties and cofactor requirements have been described (1). The decarboxylase does not attack tryptophan, 7-hydroxytryptophan, 5-hydroxy-D-tryptophan, and a variety of other amino acids. It can be separated from DOPA decarboxylase.

When 5-hydroxytryptophan is administered intravenously to dogs, a large portion of it is excreted as 5HTA (Table 2).

TABLE 2

FATE OF 5-OH-TRYPTOPHAN IN THE DOG

152  $\mu$ M. of 5-OH-DL-tryptophan administered intravenously.

5-OH-indoles in Urine	$\mu$ M.
Total	132.0
5HTA	15.8
5HIAA	22.3
Unidentified	93.9

The production of N-methylated 5-hydroxytryptamines takes place to a large extent in toads and in certain invertebrates in vivo. Evidence has also been obtained for the occurrence of basic 5-hydroxyindoles other than 5HTA in mammals. N-Dimethyl-5HTA, bufotenine, has recently been isolated in large amount from plants (7) and from fungi (9). The mechanism and intermediates of the methylating reaction are not as yet known. Experiments in this laboratory indicate that C<sup>14</sup>-labelled 5-hydroxytryptophan is converted to labelled dehydrobufotenine in the toad *Bufo marinus*.

When 5HTA is incubated aerobically with kidney or liver homogenates it is rapidly metabolized. This metabolic activity can be inhibited by isopropylisonicotinyl-hydrazide and is in other ways similar to that of monoamine oxidase. One of the products of oxidation is 5-hydroxyindoleacetic acid, which accounts for about 30 per cent of the metabolized 5HTA. In the presence of semi-



carbazine no 5-hydroxyindoleacetic acid is formed, and a new indole spot can be demonstrated chromatographically. This is apparently the semicarbazone of the aldehyde. The remaining 70 per cent of the metabolized serotonin cannot yet be accounted for as any known compound.

When serotonin is administered to dogs, about 20 to 30 per cent of the dose is excreted as 5-hydroxyindoleacetic acid (5HIAA). Several unidentified indoles are also excreted. This acid has now been found in normal urine. It was identified by its chromatographic behavior, by distribution coefficients between various solvent systems, and by characteristic reactions for 5-hydroxyindole compounds.

A specific colorimetric method for urinary 5-hydroxyindoleacetic acid was developed, based on reaction with  $\alpha$ -nitroso- $\beta$ -naphthol, after suitable extraction. Results of analyses indicate that dogs normally excrete approximately 2 mg. of 5HIAA and human beings about 7 mg. per day. Administration of 5-hydroxytryptamine or 5-hydroxytryptophan resulted in a large increase in urinary 5HIAA. Administration of large quantities (3-5 gram) of L-tryptophan to dogs produces a barely significant rise in urinary 5HIAA. When 5HIAA was administered to dogs it was almost quantitatively excreted in the urine unchanged. One may calculate that the 7 mg. of urinary 5HIAA in human beings indicates at least 20 mg. of 5-hydroxytryptamine metabolized. This represents approximately 3 per cent of the dietary intake of tryptophan. Experiments with  $C^{14}$ -tryptophan in rabbits also indicate a rapid turnover of 5-hydroxytryptamine. The half-life of the labelled 5-hydroxytryptamine which results is less than 2 days in blood platelets. That found in stomach and intestine appears much shorter.

Although the normal functions of 5HTA are not yet known, it has been shown to have many interesting pharmacological effects. It is a potent vasoconstrictor. Its presence in blood platelets may serve as a primary hemostatic mechanism before clotting takes effect. It has potent pressor, depressor, and antidiuretic activities.

Reports from many laboratories indicate that 5-hydroxytryptamine may play an important role as a neurohumoral agent. Florey and



Florey (3) report that this substance is present in large amount in crustacean nerve and that these nerves are very sensitive to it. It is also found in mammalian brain. Several drugs which have been shown to induce marked neurological changes have been shown to be potent serotonin antagonists in vitro. These include lysergic acid diethylamide (4), medmain (10), and yohimbine (6). Recent experiments by Evarts and collaborators (2) indicate that small doses of N-dimethylserotonin, bufotenine, can cause marked neurological changes in monkeys and dogs.

Whatever the function of 5HTA may be, it appears that an appreciable fraction of the daily intake of L-tryptophan in animals is metabolized along the 5-hydroxyindole pathway.

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## DISCUSSION

DR. YANOFSKY: I believe Dr. Hayaishi stated that 7-hydroxytryptophan does not replace niacin for the rat and is inactive in the mammalian tryptophan peroxidase system. I would like to mention that in *Neurospora* 7-hydroxytryptophan effectively supports the growth of a niacin auxotroph which is blocked in the conversion of kynurenine to 3-hydroxykynurenine. Presumably the 7-hydroxytryptophan gives rise to 3-hydroxykynurenine without first being converted to kynurenine, since kynurenine will not support the growth of this strain.



## SUMMARY

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IT MUST BE admitted that the treatment of amino acids in many extensively used textbooks of biochemistry is woefully inadequate and misleading. There are generally said to be 19 (or 21), or maybe about 25, naturally occurring amino acids, although the number now known actually exceeds twice that many. Very little is said about the synthesis of amino acids, only generalities are stated in regard to protein synthesis, and no overall view of the reactions of amino acids leading to the production of other amino acids, of peptides, of excretory products, or of other compounds is supplied. Of the "general reactions" of amino acids, deamination receives the fullest recognition, probably because of its importance in the formation of ammonia and the ultimate production of urea. Decarboxylation is scarcely mentioned; transamination and transmethylation are beginning to be regarded as possibly of some future importance; while peptide and protein syntheses are customarily honored with a few generalities. A few works have begun to portray the field more adequately and to orient students in this obviously key area. Yet the time is surely ripe for a major revision and reorganization of our thinking about amino acid metabolism. The current McCollum-Pratt Symposium has undertaken to lay the basis for that.

In the introductory session, which was devoted to general considerations about amino acid metabolism, Alton Meister opened with discussions of deamination, decarboxylation, and transamination. This was followed by a description of modes of amino acid transport, by H. N. Christensen, and of the amino acid pool and its relation to the formation of adaptive enzymes, or, as they are



now preferably called "inducible" enzymes, by S. Spiegelman. E. F. Gale rounded out this general orientation with a consideration of the problems of peptide and protein synthesis from amino acids.

### *Deamination, Decarboxylation, and Transamination*

Meister reviewed our present knowledge of the D- and L-amino acid oxidases and their specificities. Nearly 20 years ago Krebs observed the oxidation of both D- and L-amino acids in the same preparations and went on to show that this depends upon the presence of separate enzyme systems with opposite optical specificities. It is, as Meister said, curious that animal tissues, such as kidney, have a high D-amino acid oxidase activity, but that the L-amino acid oxidase activity of those tissues is relatively low. It consequently appears that the L-amino acids are chiefly deaminated by transamination reactions with  $\alpha$ -ketoglutarate, rather than by simple deamination, the resulting glutamic acid being thereafter deaminated by glutamic dehydrogenase. Only in snake venoms and tissues and in certain molds does one find high L-amino acid oxidase activity. Is it merely a coincidence that these L-amino acid oxidases utilize flavin adenine dinucleotide as coenzyme, like the highly active D-amino acid oxidase system of animal liver and kidney, whereas the weak L-amino acid oxidase system of mammalian tissues possesses riboflavin phosphate as coenzyme? At any rate, it is abundantly clear that the relative susceptibility of various amino acids to oxidation varies considerably with different enzymes. The L-oxidase of *Neurospora* shows very high activity on alanine,  $\alpha$ -aminoadipic acid, and ornithine, which are scarcely attacked at all by other L-oxidases. The L-oxidase of rattlesnake venom is much more active in deaminating the aromatic amino acids than are the other L-oxidases. The L-oxidase of rat kidney has a strong activity on proline, which is not attacked by the two other L-oxidases.

It was formerly thought that D-amino acids did not exist in nature. In fact, Pasteur regarded the occurrence of only one optical isomer of each organic compound as "maybe the only sharp differentiation between the chemistry of dead and living matter which can be made at present." Yet now a number of D-amino acids are known



to occur in nature. At least in certain cases they are formed from the L-isomers by the action of racemases. Is the function of the D-amino acid oxidases to destroy whatever D-amino acids are ingested or are formed accidentally? Meister points out that in general the utilization of various D-isomers, rather than their destruction, may constitute a better reason for the existence of these enzymes. D-Alanine is actually better than its enantiomorph in supporting the growth of some microorganisms. The combined action of D-amino acid oxidase and the L-specific transaminases may make many D-enantiomorphs utilizable. At any rate, from the point of view of the biochemist, the D-amino acid oxidase is very useful, since it permits the determination of many amino acid isomers to 1 part in 1000 or even in 10,000 of the enantiomorph. The pure isomers can also be prepared from racemic mixtures by selective destruction of one form through the action of the proper oxidase; and the oxidases are also useful in preparing from amino acids the corresponding  $\alpha$ -keto acids.

The decarboxylation of L-amino acids in all groups of organisms appears to follow the equation



Among the interesting reactions of this type one may note (1) the decarboxylation of aspartic acid in *Clostridium welchii*, in which the  $\beta$ - rather than the  $\alpha$ -carboxyl is attacked; (2) the decarboxylation of  $\alpha,\epsilon$ -diaminopimelic acid to L-lysine in *Escherichia coli*; and (3) the decarboxylation of tryptophan, which does not occur directly but follows the prior conversion of tryptophan to 5-hydroxytryptophan. In all known cases, with the possible exception of histidine decarboxylase, pyridoxal phosphate functions as coenzyme in these decarboxylations. The amines which are produced by these reactions are often of considerable physiological importance, viz., taurine, histamine, serotonin (5-hydroxytryptamine); and possibly  $\gamma$ -aminobutyric acid, derived from glutamic acid, in the brain. Most decarboxylations are not appreciably reversible, but possibly in some cases a synthesis of amino acids from  $\text{CO}_2$  and amines might occur.

The study of transaminations, to which Meister himself has contributed so substantially, has grown apace in the past few years.



The work may be said to have begun long ago (1909-10) with the work of Neubauer and Knoop, but it was not until 1937 that the reaction was shown to be enzymatic. Once thought to be limited to a very few amino acids (aspartic acid, glutamic acid, alanine), transamination is now known to involve many—perhaps even all—amino acids and to provide in animals and microorganisms for rapid incorporation of exogenous amino acid nitrogen into the other amino acids. Transamination also permits the  $\alpha$ -keto analogues to be utilized for growth.

The glutamate-aspartate and glutamate-alanine reactions, the first-known and the commonest transaminations, are each catalyzed by a specific enzyme. They may be combined in certain tissues to produce an aspartate-alanine transamination system; and many other such complex and also reversible systems have been demonstrated to occur in microorganisms and animal tissues. In liver preparations glutamine is converted to  $\alpha$ -ketoglutarate and ammonia by a transamination step followed by deamidation; and an analogous reaction converts asparagine to oxaloacetate and ammonia. The spectrum of the transaminase systems is very broad, for more than 30  $\alpha$ -keto acids have been found to react in the glutamine system alone. Other transaminations have been found that do not involve glutamine or glutamate, asparagine or aspartate, or their keto analogues, e. g., ornithine-pyruvate transamination in liver or kidney preparations, transaminations between monocarboxylic acids in *E. coli*, and leucine-pyruvate transamination in *Brucella abortus*. The ornithine-pyruvate and certain analogous transaminations yield the corresponding amino acids and glutamic  $\gamma$ -semialdehyde. It is, strikingly, the  $\omega$ - rather than the  $\alpha$ -amino group of ornithine that is transferred.  $\gamma$ -Aminobutyrate,  $\beta$ -alanine, and possibly lysine undergo transamination of the  $\omega$ -amino group.

There are probably many specific transaminases, although few of them have been isolated and purified as yet, apart from the glutamate transaminases already mentioned. Meister and his co-workers have fractionated transaminase systems from *E. coli*, and also found that a mutant with an absolute requirement for L-isoleucine and a relative requirement for valine, neither of which could



be supplied by the  $\alpha$ -keto analogues, could be explained by a single block of the valine, isoleucine/glutamate transaminase reaction together with persistence of the weaker valine/alanine,  $\gamma$ -amino-butyrate transaminase system. Inasmuch as the few transaminases already isolated have rather specific substrate requirements, it may be anticipated that in the future more and more of the transaminase reactions will be found to have specific enzymes.

This is borne out by the contribution of Thorne, who reports that sonic extracts of *Bacillus subtilis* contain transaminases specific for certain D-amino acids. D-Glutamic acid was synthesized from  $\alpha$ -ketoglutaric acid and either D-aspartic acid or D-alanine, and less actively from D-methionine or D-serine. D-Alanine was produced from pyruvic acid and D-aspartic acid or D-glutamic acid. Alanine racemase interconverted the two alanine isomers, and thus supplied a general path leading from the L-amino acids to D-glutamate. The discovery of these D-transaminases reinforces what was said earlier about the metabolic importance of the D-amino acids.

Snell's suggestion that pyridoxal and pyridoxamine, which can be interconverted by a nonenzymatic transamination with glutamic acid and  $\alpha$ -ketoglutarate, might well play a role in the enzymatic transamination systems, now appears to be fully established. Vitamin B<sub>6</sub> deficiency interferes with transaminations, and the purified enzymes are activated by pyridoxal phosphate. The effect of the B<sub>6</sub>-deficiency on transamination may be removed by adding pyridoxal phosphate and pyridoxamine phosphate to the enzyme systems. The original failure to find that pyridoxamine phosphate can reactivate the pig heart glutamate-aspartate apotransaminase system has now been corrected by Meister and coworkers through the use of the crystalline coenzymes. The pyridoxamine phosphate merely requires somewhat longer to combine with the apoenzyme. The evidence suggests that the interconversion between the forms of the coenzyme must take place in the presence of substrate after the enzyme and coenzyme have combined. Deoxypyridoxine has been found to be a very active B<sub>6</sub> antagonist. It acts, according to Meister, by competing with the coenzymes for the enzyme.

So far, however, the experimental demonstration of the conversion



of enzyme-pyridoxal phosphate to enzyme-pyridoxamine phosphate or of the reversibility of the reaction has not been successful. Pyridoxal-pyridoxamine phosphate is of course the coenzyme not only for the transaminase systems but also for amino acid decarboxylations and many other amino acid reactions, and vitamin B<sub>6</sub> may thus be characterized as *the* vitamin of amino acid metabolism. Transamination is the initial step in the degradation of many amino acids, is an essential step in the biosynthesis of some, and is probably the key to the utilization of the  $\alpha$ -keto acid analogues of the amino acids in the growth of animals and microorganisms. Its widespread occurrence in all organisms signifies its importance to the interconversions of the amino acid pool. By linkage with decarboxylation and dehydrogenase systems it ties into amine and carbohydrate metabolism. It is surely of general significance in nitrogen metabolism.

Some very interesting examples of stereospecificity and of interference between isomers were reported by Altenbern as a result of studies on the shifts in *Brucella* populations from smooth to nonsmooth colony-formers. The general pattern of reactions studied leads up to the synthesis of pantothenic acid and the maintenance of the smooth type, and is diagrammed in Fig. 1. Stereospecific asparaginases respectively deamidate L- and D-asparagine without mutual interference; but D-asparagine interferes with the conversion of L-asparagine to  $\beta$ -alanine (reaction A). Again, pantoate, derived from either isomer of valine, is coupled with  $\beta$ -alanine to produce pantothenic acid, and this reaction (B) is markedly stimulated by L-serine by means of some mechanism that is blocked by D-serine. The coupling of pantoate and  $\beta$ -alanine is also blocked by L-leucine or L-isoleucine. Altenbern concludes that the synthesis and utilization of pantothenate in *Brucella* is a particularly sensitive part of the metabolism, and that either the presence of inhibitory amino acid isomers or the insufficient synthesis of those amino acids directly involved may lead to selective conditions that result in the replacement of the smooth by the now fitter nonsmooth type.

Umbreit reported a new approach to the elucidation of amino acid reactions, through the preparation and study of amino acids with



an  $\alpha$ -methyl substitution. This approach seemed particularly promising because the alteration is structurally quite minor, and one of the natural amino acids, alanine, may be described as an  $\alpha$ -methyl substituted glycine.  $\alpha$ -Methylglutamic acid was studied quite fully.

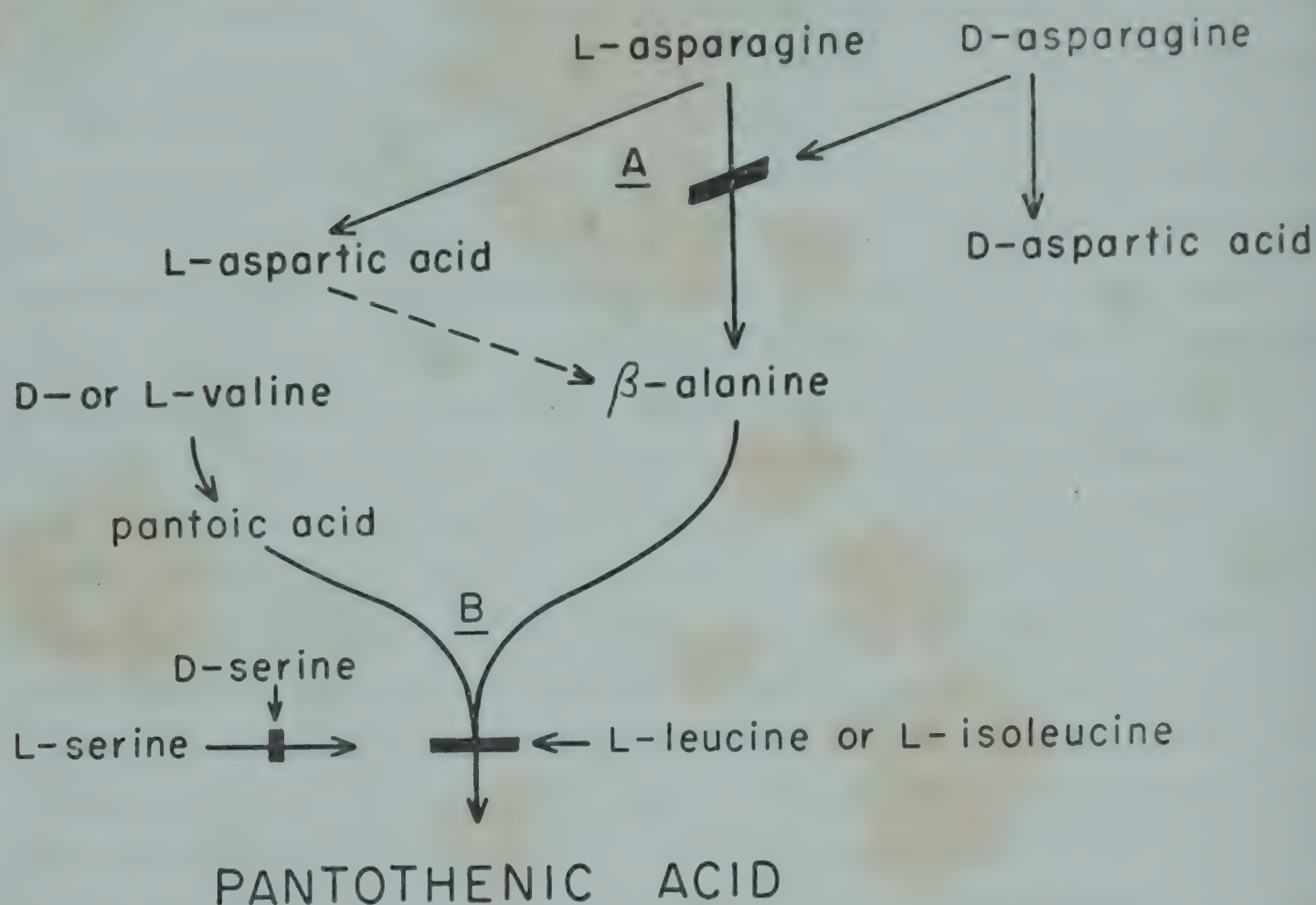


FIG. 1. The synthesis of pantothenic acid in *Brucella*, according to Altenbern. The bars across arrows represent the existence of mutants that block the respective reactions, and side-arrows pointing to the blocks represent the inhibition of the reaction by the respective compounds. The arrow from L-serine represents stimulation of reaction B.

Transamination and dehydrogenation reactions, which obviously involve the  $\alpha$ -hydrogen atom, were, as might be expected, interfered with, but it was surprising to find that decarboxylation, which does not involve the  $\alpha$ -hydrogen, was also inhibited.  $\alpha$ -Methylglutamic acid served readily as a substrate for the synthesis of ( $\alpha$ -methyl) glutamine, but inhibited the breakdown of glutamine and the activity of glutamotransferase.  $\alpha$ -Methylalanine,  $\alpha$ -methylvaline, and  $\alpha$ -methylserine mostly proved to be inert, but  $\alpha$ -methylphenylalanine and  $\alpha$ -methyltryptophan present an interesting contrast, for the former does not inhibit the oxidation of phenylalanine, whereas the latter blocks the oxidative degradation of tryptophan to kynure-



nine, although it is itself oxidized by the same pathway.  $\alpha$ -Methyldihydroxyphenylalanine ( $\alpha$ -methyl DOPA) completely inhibits DOPA decarboxylase when at a high concentration, but at a lower one it actually increases the enzyme's activity. It combines slowly and reversibly with the apoenzyme.

### *Amino Acid Transport into Cells*

The first step in the metabolic utilization of an amino acid is, as Christensen points out, its transport through the cell membrane into the interior. The intracellular concentration averages less than 10 times the extracellular level. Of course, being against a concentration gradient, the transport requires some energy. This, however, constitutes only a small part of the total energy of protein synthesis. Christensen's own studies have utilized in particular the rat diaphragm, mammalian erythrocytes, and the neoplastic cells of the Ehrlich mouse ascites tumor. The concentration of glycine in the case of the erythrocytes, and of both glycine and tryptophan in the case of the ascites cells attains a steady state distribution, and the rate of inflow (for glycine) shows that the amino acid must become bound to some acceptor in the rate-limiting step. The inflow is not slowed down by the prior presence of glycine in the cell, although during efflux the initial rate is proportional to the apparent level of glycine previously accumulated in the cell. A striking temperature dependence of the transport was evident,  $Q_{10}$  being 1.8 in the range between 24° and 32° C. Cells that have been broken up fail to accumulate amino acids, a fact perhaps related to their potassium loss, for high external potassium (one-half the cellular concentration) also prevents the uptake, and a high influx of the amino acids leads to a corresponding exodus of potassium. If all the amino acids were accumulated to the same level as glycine and  $\alpha,\gamma$ -diaminobutyric acid, individually, and if all remained bound in the cell, the effective binding agent or carrier would have to amount to about 10 per cent of the cell's total solids. This is a strong argument in favor of the view that the amino acids are set free after entering the cell. From the direct proportionality of the water uptake to the glycine uptake in the ascites tumor cells, Christensen concludes that the amino acids



exist in a free state within the cells, inorganic ions having been excluded as a factor in the water uptake. At high concentrations, the entering glycine more than compensates for the lowering of osmotic pressure due to the loss of the potassium. The intracellular amino acids retain both their osmotic activity and their net charges. It thus seems most likely that the amino acid is promptly freed after being introduced into a cell.

The accumulation of glycine, tryptophan, diaminobutyrate, and methionine is stimulated by pyridoxal and its analogue, 4-nitrosalicylaldehyde; but it is not so greatly affected by pyridoxal phosphate, and not at all by pyridoxamine or pyridoxine. These observations make it seem likely that in this case the role of pyridoxal is not that of a coenzyme in the usual sense, but rather that the amino acid may combine with pyridoxal or some derivative of it as a carrier. Inhibitors of respiration or oxidative phosphorylation do not interfere regularly with amino acid accumulation, but do so only in certain cells and scarcely at all in others. In the ascites tumor cell, both L- and D-isomers of the amino acids are accumulated, although the former are usually more strongly concentrated. An increase in length of the aliphatic hydrocarbon sidechain or the presence of a second carboxyl group decreases the degree to which an amino acid is accumulated, and an increase in the number of electron-attracting groups in the sidechain or the presence of a methyl group on the  $\alpha$ -carbon, or of a second amino group, increases it. Christensen favors the view that the formation of a Schiff's base between the amino group and a pyridoxal derivative is the most likely mechanism of transport. The source of energy for this might come either from the splitting of a compound of higher energy in the formation of the carrier—amino-acid complex, or from some intracellular change in the complex, such as phosphorylation, that would increase its energy content after it had entered. The central question raised by Christensen is whether, on its way to protein synthesis, an amino acid has to pass through the pool of free amino acids, or whether, alternatively, the free amino acid pool lies to one side of the direct stream of synthesis. The two alternatives are represented in Fig. 2, whence it will be noted that if the free amino acid pool is on



the direct path (alternative 2) the amino acids must undergo a second activation, distinct from the binding with the carrier during transport across the cell membrane. In any event, the other reactions into which amino acids enter, the transaminations, deaminations, syntheses of non-protein compounds such as heme, creatine, and purines, etc., must be intimately connected with the pathway of protein synthesis,

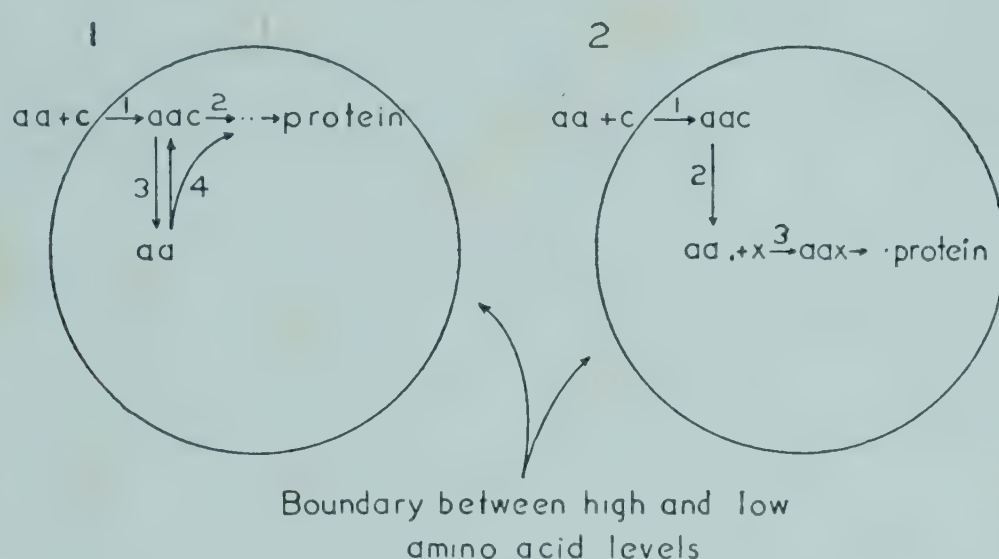


FIG. 2. Schematic representation of possible relationships between the amino acid transfer process and protein synthesis. Alternative 1. The amino-acid—carrier complex donates the amino acid to other acceptors functioning in protein synthesis. Alternative 2. The amino acid must be released and recombined before proceeding into protein synthesis. (From Christensen).

for the degradation of amino acids to supply energy and for other uses takes precedence over the protein syntheses, and the supply of free amino acids in the cell is not depleted either by protein synthesis or starvation.

Certain striking differences exist between the behavior of the ascites cells studied by Christensen on the one hand, and yeasts and bacteria, on the other; and in discussion Gale emphasized that it is probably a mistake to expect all types of cells to exhibit the same transport mechanisms, at least superficially. Also, as to the relation of the free amino acid pools to protein synthesis, in the staphylococci the amount of free amino acid (glutamic acid) is reduced during the synthesis of proteins and becomes greatest when protein synthesis ceases; whereas the converse is true for amino acids in the mouse ascites tumor cells. During protein synthesis the entering amino acids appear to go promptly and quantitatively into the new protein



in the former case, but in the latter they enter the free amino acid pool more rapidly than they enter the protein. Halvorson and Spiegelman pointed out that the studies on yeast imply that utilization of the free amino acid pool is obligatory in protein synthesis. This would favor Christensen's alternative scheme 2, but the conflicting data from the staphylococci and the tumor cells impress one, in Gale's words, "that the process concerned in controlling the concentration of a given amino acid in the pool differ widely from organism to organism."

### *The Amino Acid Pool*

Spiegelman and his coworkers presented important evidence about the significance of the free amino acid pool in protein (i. e., in enzyme) synthesis, and they reported also the existence of a nucleotide pool which is equally essential to the same process. Their studies were made on yeast cells and on *Escherichia coli*. The primary question that concerned them was the nature of the precursor material which becomes transformed into active enzyme, and the primary datum was the ability of such cells, especially the yeasts, to synthesize an enzyme in the absence of any external supply of nitrogen. It was this last-mentioned finding that led to the demonstration of the existence of a free amino acid pool within the cells (Taylor). In studying the composition of the free amino acid pool, Spiegelman and his collaborators have used a simple boiling procedure to produce disrupted cells, after the method of Gale. The pools were readily demonstrable in many strains of yeast and included at least 16 different amino acids, of which glutamic acid, aspartic acid, and serine were the principal ones. The level and composition of the pool could be altered by depleting the cells of their nitrogen and then replenishing it; but exposure of the cells to a nitrogen-free medium containing a utilizable energy source does not guarantee that the internal nitrogen supply in the amino acid pool will disappear. It may lead instead to an accumulation of amino acid nitrogen, and this observation implies the existence of some internal mechanism for replenishing the pool. The probable source of replenishment is some labile protein, for it replenishes



all the amino acids, whereas a single amino acid supplied to cells with a depleted amino acid pool cannot restore the pool fully. In discussion, Kallio mentioned that in the aerobic bacteria *Pseudomonas fluorescens* and *P. aeruginosa* no amino acid pool could be demonstrated, but keto acids and ammonia supplied its place. This seems to be true of all gram-negative bacteria (Taylor).

In studies of the synthesis of enzymes in yeast, Spiegelman and Halvorson found that analogues of certain amino acids will effectively prevent not only the incorporation of those acids into the protein fraction of the cell, but the incorporation of nearly all the other amino acids as well. The first stable intermediate on the way to the synthesis of an enzyme molecule must therefore be a compound of sufficient complexity "to demand the simultaneous utilization of all the amino acids." Moreover, in the study of the synthesis of  $\beta$ -galactosidase in *E. coli*, tracer experiments with  $C^{14}$ -labeled lactate revealed that less than 1 per cent of the carbon incorporated in the newly formed enzyme could have come from any components present in the cell prior to the induction of the enzyme synthesis. (Under these conditions the internal mechanism for replenishing the free amino acid pool is in abeyance.) Similar results with the tracer  $S^{35}$  were obtained by Cohn and Hogness. Thus it seems clear that the enzyme must be synthesized de novo from free amino acids, and such protein synthesis naturally suggests a template mechanism. Steinberg pointed out in discussion that a template mechanism need not imply that synthesis of the protein molecule could not be step-wise. It need not occur by simultaneous condensation of all the amino acids on the molecular model, and if the amino acids are absorbed in a series of steps, then the specific activities of various amino acid residues of the same kind, e. g., glycine, need not be identical. This was actually found to be the case in experiments conducted in Anfinsen's laboratory; and the longer the time allowed for synthesis (i. e., the longer the incubation period), the less became the differences between the labeling of differently located amino acid residues of the same kind.

If, as Spiegelman asserts, any template must be as large and as complicated in structure as the molecule it forms, this restriction



limits consideration of template structures to (a) desoxyribose nucleic acid, (b) ribose nucleic acid, and (c) protein—or complexes of these. Spiegelman et al. tested the roles of these substances in enzyme formation in *E. coli* and yeast. In both organisms the synthesis of DNA can be blocked without causing much alteration in the formation of inducible enzymes. On the other hand, even a 50 per cent reduction of RNA synthesis, produced by means of ultraviolet irradiation, completely abolished the synthesis of the enzyme in yeast. In *E. coli*, this was also halted by purine and pyrimidine analogues, such as 5-hydroxyuracil. Mutants of *E. coli* unable to synthesize uracil or adenine could synthesize no enzyme until supplied with those compounds; but a thymine-requiring mutant synthesized the enzyme successfully. These results clearly indicate that ribose nucleic acid, and not desoxyribose nucleic acid, is involved in the specific formation of  $\beta$ -galactosidase in the test organisms.

Far from enhancing the synthesis of  $\beta$ -galactosidase, an external supply of amino acids immediately stopped it in *E. coli*. This could be circumvented, it was found, by adding an extra supply of ribose nucleic acid precursors and preincubating before adding the amino acids. There was a temporary loss of the ability to synthesize the enzyme by mutants requiring uracil or adenine, when grown at limiting amounts of the respective pyrimidine or purine.

In yeast quite the contrary happened. The purine and pyrimidine analogues had no inhibiting effect on the synthesis of  $\beta$ -galactosidase, and purine or pyrimidine-requiring mutants synthesized it as readily in the absence of those bases as in their presence. This led to the demonstration of an internal supply of nucleotides in the yeast cell, a nucleotide pool comprised mainly of adenylic acid, guanylic acid, and uridine, and lacking the free bases. Depletion and replenishment of the pool was accomplished, and it became possible by this means to test the role of nucleotides in enzyme synthesis. The amount of synthesis of  $\alpha$ -glucosidase in yeast exactly paralleled the changing level of the nucleotide pool. That the nucleotide pool is indeed necessary for enzyme synthesis was shown by experiments from which it was concluded that new molecules of  $\alpha$ -glucosidase are formed only if and when new nucleic acid is synthesized. Cells



that have been depleted of nucleotides cannot synthesize more enzyme, although their enzyme content may be fairly high. But cells with a replenished nucleotide pool synthesize more enzyme immediately upon being exposed to the inducer. In short, "the synthesis of new nucleic acid is a compulsory concomitant of the continued formation of new enzyme molecules."

### *Protein and Peptide Synthesis*

In considering the synthesis of proteins, a topic of paramount interest, Gale explicitly limited himself to an appraisal of the mechanisms whereby amino acids are incorporated into protein in *Staphylococcus aureus*. Nevertheless his contributions, added to those of Christensen and Spiegelman et al., serve to bring out principles that seem likely to have very broad and general importance. In staphylococci, unlike yeast cells, the amino acid is removed from the external medium and transported into the cell at a much greater rate when the amino acid is entering the free amino acid pool than when it is being incorporated into protein. When the latter process is occurring, the amino acid is removed from the medium and embodied in the protein at equivalent rates. If the bacteria are supplied with a single amino acid and a source of energy, there is no increase in protein, but nonetheless the amino acid can by labeling be shown to become incorporated in the protein. The study of this process in disrupted cells renders it clear that exchange reactions can occur between the amino acid residues of the protein and the free amino acid. This exchange reaction can be abolished by removal of nucleic acid, and restored by either the DNA or the RNA from staphylococci, although not by that from other sources. In the case of glutamic acid, the incorporation ceases when the amount taken up is only a small portion of the total amount of glutamic acid residues in the preparation; hence the exchange is probably limited to a particular type of protein, perhaps the nucleoproteins. The effect of nucleic acid varies with the particular kind of amino acid entering into the process, being highest with glycine and non-existent with alanine.

Gale's report of the successful synthesis of three enzyme systems



in disrupted staphylococci aroused considerable excitement. The disrupted cells were incubated with ATP, hexose diphosphate, a complete mixture of amino acids, and either RNA, DNA, or a mixture of purines and pyrimidines. Of the three enzyme systems, that producing acid from glucose ("glucozymase") is complex, and led to less certain conclusions than the others; but it is interesting that synthesis of this system is more strongly affected by a medium containing a mixture of purines and pyrimidines than by either DNA or RNA, although all three were very effective. Catalase, the second enzyme produced by the disrupted cells, is especially actively synthesized when RNA is added to the medium, and the purine-pyrimidine mixture had little or no effect on it. Synthesis of the third enzyme,  $\beta$ -galactosidase, was doubled or tripled by the purine-pyrimidine mixture, was affected by DNA in highly resolved preparations, but never responded to RNA. The disrupted cells at the same time synthesize RNA from the purine-pyrimidine mixture, and the synthesis of the enzyme is abolished by ribonuclease. Labeled uracil was incorporated 5 or 6 times as fast when galactose was added to the medium as inducer of  $\beta$ -galactosidase; but thymine was not found to be incorporated under any circumstances. From these results it would appear that RNA plays a most important role in the elaboration of at least certain proteins (catalase,  $\beta$ -galactosidase). Nonetheless, DNA is more often effective than RNA, and, since its effect is abolished by the action of ribonuclease, it may be that DNA "organizes RNA synthesis and . . . provides the initial model on which, first RNA, then protein, synthesis takes place."

Gale regards the following scheme as in accordance with all of the present experimental evidence. Desoxyribose nucleic acid begins the process by combining with individual amino acids the positions of which are determined by specific combinations of nucleotides in the DNA with specific amino acids. The amino acids cannot combine with each other at this stage, but must be first taken over by a ribose nucleic acid with the corresponding structure. This must often entail a synthesis of the right RNA molecule from nucleotide residues [perhaps drawn from the nucleotide pool of Spiegelman?]. The RNA molecule, acquiring the right specificity from the DNA-



protein complex, permits its attached amino acid residues to combine by peptide linkages, while still in the positions ordained by the DNA template. When completed, the polypeptide peels off from the RNA molecule. Such a scheme—which those interested may like to compare with Fritz Lipmann's model of polypeptide synthesis in the Symposium last year—will explain why the synthesis of ribose nucleic acid requires a full assortment of amino acids and why penicillin, which prevents amino acid exchanges, will inhibit both the synthesis of the protein and that of RNA. From Lipmann's scheme, taken in conjunction with this one of Gale, the thought arises that failure of the polypeptide to form on the DNA molecule and its ability to form on the RNA molecule may arise from the failure of DNA and the converse capacity of RNA to react at the specific combining sites for amino acids with the high-energy compound required for activation of the amino acids. Whatever scheme is proposed as an alternative to this one will in any case have to reckon with the experimental demonstration that the exchange between free amino acids and their corresponding residues within protein molecules, and also the synthesis of proteins from free amino acids, involve "a confluence of those amino acids and those proteins and the nucleic acids of the cell."

Bonner's observations on that very popular enzyme,  $\beta$ -galactosidase, added two additional points of great interest. Firstly, in diauxic growth (i. e., growth on two substrates one of which is used before the other), the synthesis of  $\beta$ -galactosidase continues at a constant and maximum rate during the lag phase, while the overall synthesis of protein comes to a nearly complete halt. Taken with Gale's results, these findings that general protein synthesis is "neither a prerequisite nor a necessary characteristic of the formation of this specific enzyme" fortify the conclusion that it is the nucleic acids which play the chief roles in enzyme synthesis. Secondly, a large part of the enzyme within an intact adapted cell appears to be masked. In addition to the existence in the intact cell of a demonstrable barrier that prevents access of the substrate to the site of synthesis, which is modifiable by gene mutation, and is not affected by temperature, penicillin, or the internal concentration of



$\beta$ -galactosidase, there is a second barrier, a so-called "Factor," that, even when the first barrier is supposedly<sup>1</sup> fully compensated for by increasing the concentration of the external substrate, still manifests itself by the existence of about 15 times more  $\beta$ -galactosidase activity in extracts than in the intact cells. This second barrier is measured operationally by the ratio of the enzymatic activity of extracts to that of intact cells. In contradistinction to the first, it is affected by both penicillin and temperature, is a function of the intracellular  $\beta$ -galactosidase concentration, and varies in magnitude in a different way in adapting and in deadapting cells. In the former it increases very rapidly to nearly the maximal value, whereas in deadapting cells it is approximately halved with each cell division. Bonner suggests that the rapid increase of the "Factor" in the adapting cells is because the newly formed enzyme remains for some time bound to a cellular component, and that the probable identity of the latter is the enzyme-forming system itself. "One might visualize that, during the formation of  $\beta$ -galactosidase, enzyme molecules pile up much as shuttle-cocks are packed in cans." In Gale's suggested scheme, this would be described as a failure of the newly formed polypeptides to peel off from the specific RNA enzyme-forming molecule as fast as they are formed, and makes one wonder whether this wouldn't at once interfere with the transfer of additional amino acid groups from the DNA-template, and so bring the process of synthesis rapidly to an end. Bonner visualizes the outermost enzyme molecules as undergoing release at some limiting height of the pile and so in adapting cells becoming shifted from the masked state to active enzyme. Another point of significance is that even unadapted cells possess a small amount of  $\beta$ -galactosidase, even in the absence of the exogenous inducer. Further elucidation of the mechanism which prevents every molecule of newly formed  $\beta$ -galactosidase from becoming immediately active will be keenly anticipated.

Enzyme studies of a particular mutant of *E. coli* have led Dubnoff to a concept of the reversible activation of "adaptive" enzymes.

<sup>1</sup> The validity of this assumed compensation was seriously questioned during the discussion by Davis and Gunsalus, and vigorously defended by Bonner and Vogel.



Dubnoff found that vitamin B<sub>12</sub> will activate adaptive enzymes, such as formic hydrogenlyase, maltase, and lactase, in the complete absence of substrate. In aged cells grown on casein and originally producing formate and containing the enzyme, the latter gradually disappears. When it is almost completely gone, the addition of vitamin B<sub>12</sub> will restore a considerable amount of activity. The presence of the substrate retards the inactivation of the enzyme. When the same mutant is grown on methionine and glucose, no hydrogenlyase is observable until after activation with vitamin B<sub>12</sub>, and then CO<sub>2</sub> and H<sub>2</sub> are evolved from formate at a constant rate.

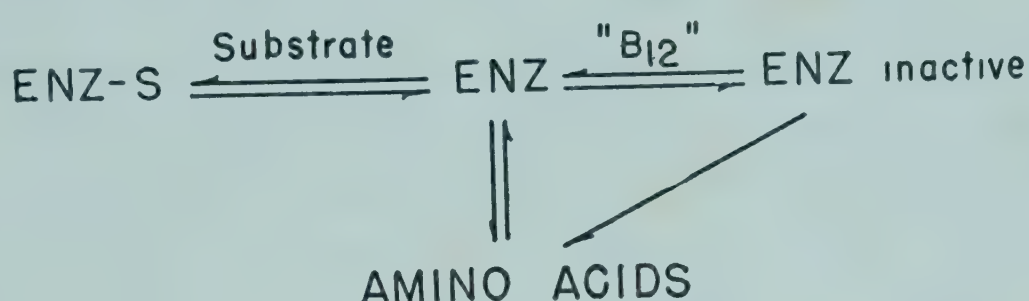


FIG. 3. Scheme to illustrate the reversible activation of an "adaptive" enzyme, according to Dubnoff. The enzyme is stabilized by combination with substrate, and otherwise goes into an inactive state from which it may be reactivated by vitamin B<sub>12</sub>. (From Dubnoff.)

The curve representing this evolution of gas from the substrate passes through the origin and demonstrates that the enzyme has been formed in the absence of the substrate. Enzyme never exposed to formate is inactivated at a much higher rate than that which has been exposed. Dubnoff concludes that the enzyme is not induced by the substrate but is simply stabilized by it. In other words, an organism synthesizes all the enzymes within its genetic capacity, but some are stable and some labile except in the presence of their own substrates. Deactivation is then a consequence of the instability of the enzyme in the absence of its substrate. In discussion, Dubnoff stated that he saw no contradiction between his interpretation of the nature of the inactive enzyme and Spiegelman's demonstration that new enzyme is made almost exclusively from non-protein carbon and sulfur. The synthesis of new enzyme molecules is not equivalent to the reactivation of the enzyme from an inactive, degraded product of the enzyme—the inactivated form is not necessarily an intermediate in the synthesis of the enzyme (Fig. 3).



Horowitz has put forward a suggestive hypothesis of an enzyme synthesis by means of an autocatalytic reaction in which the enzyme itself might be described as a byproduct. Extracts from young adult *Drosophila* contain no detectible tyrosinase, but upon standing at 0° C. the presence of the enzyme is readily detected colorimetrically when either tyrosine or dopa is used as substrate. There is also an inhibitor of tyrosinase in the fresh extract. The activation phenomenon is a bimolecular reaction; that is, it involves a tyrosinase precursor and an activator. Experiments with various inhibitors and substrates clearly showed that the inhibition of activation and the inhibition of tyrosinase activity do not run parallel, and tests in which active tyrosinase was added to fresh extracts demonstrated that it could not itself be the activator, although the conception of the activator also increases during activation. The simplest explanation is that described by the equation:



This describes an autocatalytic reaction on the part of the activator in which tyrosinase is a simple product. Such a novel type of reaction will require further study before the hypothetical mechanism can be regarded as really established.

Williams and Thorne added a different type of contribution to the consideration of protein synthesis by appending a postscript to the study of  $\gamma$ -glutamyl transfers reported by Waelsch at the McCollum-Pratt Symposium two years ago. *Bacillus subtilis* was found to yield an enzyme preparation that catalyzed the transfer of the  $\gamma$ -glutamyl radical from L-glutamine to D-glutamic acid,  $\gamma$ -D-glutamyl-D-glutamic acid, aspartic acid, or perhaps to glutamine itself, to form glutamyl dipeptides and tripeptides. The same preparation also catalyzed a transpeptidation reaction which could proceed almost entirely as a transfer reaction without any required cofactor, and in which presumably two molecules of the dipeptide yielded one molecule of free glutamic acid and a tripeptide. Further activity of the enzyme preparation yielded two still longer peptides, perhaps corresponding to a polypeptide formed by *B. subtilis*.



*The Metabolism of Glutamic and Aspartic Acids, Proline, and the Ornithine-Citrulline-Arginine Cycle (see Fig. 4)*

The five-step ornithine-citrulline-arginine, or urea, cycle in mammalian tissues, as outlined in these symposia three years ago by Ratner and by Grisolia, has in the meantime undergone certain modifications and clarifications, without being essentially altered.

Like the citrulline to arginine part of the cycle, the ornithine to citrulline part has been shown, especially by the work of Grisolia and his co-workers, to comprise two distinct steps (see Fig. 4). The first of these brings about the formation (by means of Enzyme I) of an active intermediate through the combination of carbamyl glutamate with  $\text{CO}_2$  and  $\text{NH}_3$ , in a reaction depending strongly on phosphate-bond energy. In the second step (catalyzed by Enzyme II), the active intermediate reacts with ornithine to yield citrulline. In the rat liver preparations which Grisolia has utilized for his studies,  $\text{C}^{14}\text{O}_2$  is incorporated into citrulline many times faster than into carbamyl glutamate, which is apparently required only in catalytic amounts for the cycle to proceed. Numerous N-derivatives of glutamic acid will substitute for carbamyl glutamate, both acetyl and chloroacetyl glutamate being more active than the carbamyl compound, while propionyl and formyl glutamates are less active, in that order. From a comparison of these compounds, the L-glutamic portion seems to be required specifically, and the substituted amino group must carry a carbonyl group that is capable of enolization. Any of the active intermediates will decompose spontaneously, but much more rapidly by means of enzymes, perhaps phosphatases, contained in various muscle extracts. Even "Compound X," the intermediate formed from carbamyl glutamate, has not yet been fully characterized, although it is known to contain one mole each of carbamyl glutamate,  $\text{NH}_3$ ,  $\text{CO}_2$ , and phosphate. In decomposing, the compound releases these in the reverse order, so that ammonia is held most firmly. Only the  $\text{—N—C—R}$  part of the active glutamate derivative seems to be involved in the reaction. Whether one



mate derivative seems to be involved in the reaction. Whether one



and the same enzyme can catalyze the activation of all the glutamate derivatives named above remains uncertain, although a single enzyme appears to activate both the carbamyl and formyl glutamates. In mammals, all these reactions are limited to the liver in so far as present knowledge goes.

In a number of laboratories within the past two years attention has been concentrated on the microbial reversal of the ornithine to citrulline reaction, which has proved most interesting because it revealed a new mechanism for the generation of energy-rich phosphate. The reverse reaction, obtained primarily in the streptococci, pseudomonads, and clostridia, breaks down citrulline to ornithine,  $\text{NH}_3$  and  $\text{CO}_2$ , and simultaneously generates ATP. The system was separately described in the symposium by Oginsky, Korzenovsky, and Slade, each of whom had a preferred name for the enzyme—respectively called citrullinase, citrulline phosphorylase, or citrulline ureidase.

The requirements for the reaction are acetone-treated preparations or cell-free extracts (necessary because citrulline penetrates intact cells very slowly), inorganic phosphate,  $\text{Mg}^{++}$ , and AMP or ADP. The reaction may be completely replaced by an arsenolysis which is uninfluenced by  $\text{Mg}^{++}$ , requires no inorganic phosphate, and of course yields no ATP. Carbamyl glutamate does not stimulate the arsenolysis, but both  $\text{Hg}^{++}$  ions and sodium fluoride inhibit the arsenolysis and phosphorolysis alike. Oginsky and Slade each reported on these and other inhibitions and their reversal, and Oginsky especially emphasized the inhibition by ornithine of both reactions, the phosphorolysis being the more sensitive. It thus appears that the rate of generation of high-energy phosphate by this mechanism is controlled in bacteria not only by the concentrations of the substrate and the cofactors, but also by that of the product, ornithine. Slade noted that the enzyme was inhibited by iodoacetate and by *p*-chloromercuribenzoate, inhibitions which imply the sulfhydryl nature of the enzyme.

Although workers have been looking intensively for the presence of a phosphorylated citrulline in this system, it has not turned up, and perhaps the reaction takes place entirely on the enzyme, as



Oginsky suggested. Korzenovsky proposed a mechanism in which citrulline is first split to ornithine and a phosphorylated carbamic acid on the enzyme, and the latter intermediate thereafter transfers its active phosphate group to ADP, while the carbamic acid decomposes to  $\text{CO}_2$  and  $\text{NH}_3$ . If this mechanism is correct, the same phosphorylated intermediate could also occur in the mammalian liver system; and in that case the principal difference would be the participation in the latter of carbamyl glutamate or some other glutamyl derivative as a carrier of  $\text{CO}_2$  and  $\text{NH}_3$ . The capacity to synthesize citrulline may thus depend on the presence of an efficient carrier of carbon dioxide and ammonia. Slade, on the other hand, proposed a mechanism in which the citrulline is first split by the enzyme to ornithine and the carbamyl group, which remains activated on the enzyme. A second reaction might exchange the carbamyl group, which would then decompose, for inorganic phosphate, which would thus become high-energy phosphate ( $E \sim P$ ). A final step could transfer the energy-rich phosphate to ADP.

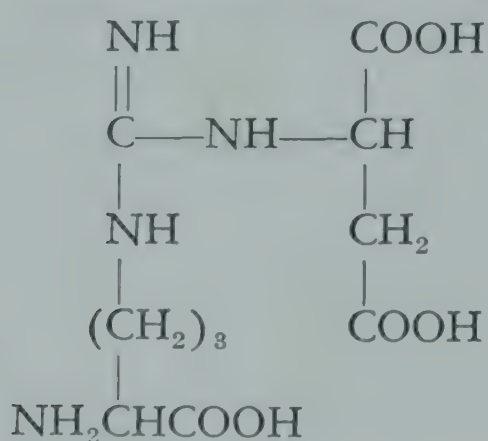
Grisolia and his coworkers have now obtained evidence that the reversal of the ornithine to citrulline reaction also occurs in mammalian tissues, but the reverse reaction is very weak compared to the forward reaction, and is probably of little importance in the mammal. Arsenolysis of citrulline to ornithine,  $\text{NH}_3$ , and  $\text{CO}_2$  also occurs in mammalian liver, and is associated with Enzyme II of the system. In the discussion Grisolia added the interesting fact that aspartic acid will substitute for ornithine as a receiver of  $\text{CO}_2$  and  $\text{NH}_3$  from Compound X—probably by means of a different enzyme, however; and Cohen referred to a study by Lowenstein, who showed that the carbamyl aspartate so formed undergoes in liver preparations an arsenolysis like that of citrulline. Levintow added that glutamine, which may be thought of as a close structural analogue of citrulline, also undergoes a reversal of synthesis that yields ammonia and is accompanied by a generation of ATP; and that this phosphorylase is likewise subject to a rapid arsenolysis.

From the work of Ratner, it has become quite clear that the source of the additional amino group acquired by arginine in its formation from citrulline is limited to aspartic acid (see Fig. 4). The two-



step reaction leads through an intermediate compound which has now been identified as argininosuccinic acid. The first step, a condensation reaction, requires ATP and  $Mg^{++}$ ; the second step is a cleavage of the intermediate to arginine and fumaric acid, rather than malic acid, as was previously thought. The particular interest of the first step lies in the difference between this condensation and others known to depend upon ATP, since the utilization of phosphate-bond energy in raising the ureido carbon of citrulline to the amidine level, and in thus leading to the synthesis of the guanidine group of arginine, is novel. The mechanism of this reaction is not yet clear. At first thought one would postulate a phosphorylation of the ureido carbon, but no evidence of the presence of a phosphorylated amino acid in the system has been obtained by Ratner.

The structure of argininosuccinic acid is probably the following:



Cleavage of this compound is but weakly endergonic ( $\Delta F^\circ = + 2800$  cal.), and may readily be reversed. Cleavage is favored at high dilutions, especially since the affinity of the splitting enzyme is about ten times as high for argininosuccinic acid as for the products of the cleavage. The specificity of the enzyme is narrow. In spite of the similarity of the cleavage reaction to the splitting of aspartic acid by aspartase to fumaric acid and ammonia, the argininosuccinic-splitting enzyme cannot split aspartic acid. Only canavanine, which is one  $\text{CH}_2$  group shorter than arginine, is able to replace arginine, and to inhibit the formation of the argininosuccinic acid. The inhibitory action of canavanine on the growth of *Neurospora* and certain bacteria is thus explicable in terms of its interference with arginine metabolism.

In bacteria the citrulline to arginine conversion is reversed. Argi-



nine is split to citrulline and  $\text{NH}_3$  by an enzyme which has been called arginine desimidase, and for which apparently no cofactors are needed. According to Oginsky, the reaction goes to completion, is not inhibited by the addition of citrulline, and must occur by means of a distinctly different enzyme than either of those that participate in the synthesis of arginine from citrulline.

In the mammalian kidney citrulline is converted into arginine by an enzyme system apparently identical to that operating in the liver, but the other parts of the ornithine cycle are absent. Since urea is not formed here, the fate of the arginine produced is of some interest. According to earlier work (Borsook and Dubnoff), guanidinoacetic acid is formed by a transfer of the amidine group from arginine to glycine, the other product being ornithine; and the guanidinoacetic acid is thereafter converted by methylation into creatine. Obviously, the arginine-synthesizing systems feed into protein synthesis; and the existence of an ornithine-citrulline-arginine sequence in many organisms (*Neurospora*, *Penicillium*, lactic acid bacteria, *E. coli*, *Tetrahymena geleii*, etc.), some of which lack arginase, emphasizes the fact that urea formation is essentially a diversion from the general synthetic mechanism whereby arginine is supplied to protein synthesis. The citrulline-aspartate condensing enzyme has now been found in yeast and *Neurospora* besides the mammalian liver and kidney, and the argininosuccinate-splitting enzyme occurs not only in all these forms but has been isolated also from *E. coli*, peas, jack beans, and *Chlorella*.

About the remainder of the ornithine cycle, the degradation of arginine to ornithine and urea by means of arginase, there is little new to be added at the present time.

Ratner has strongly emphasized the central role of glutamic acid in linking the tricarboxylic and urea cycles of Krebs (Fig. 4). Among its multiple functions is that of supplying the nitrogen which is required to synthesize arginine from citrulline and aspartic acid, inasmuch as ammonia cannot be fixed directly into aspartic acid in mammalian tissues. Through the citric acid cycle, glutamic acid also furnishes the carbon skeleton for aspartic acid by way of oxaloacetate, and furthermore serves as the energy source for the generation of



much of the ATP required in the citrulline-aspartate condensation. Hence a strong dependence of urea formation upon respiration arises in the absence of a direct supply of aspartic acid and ATP, and

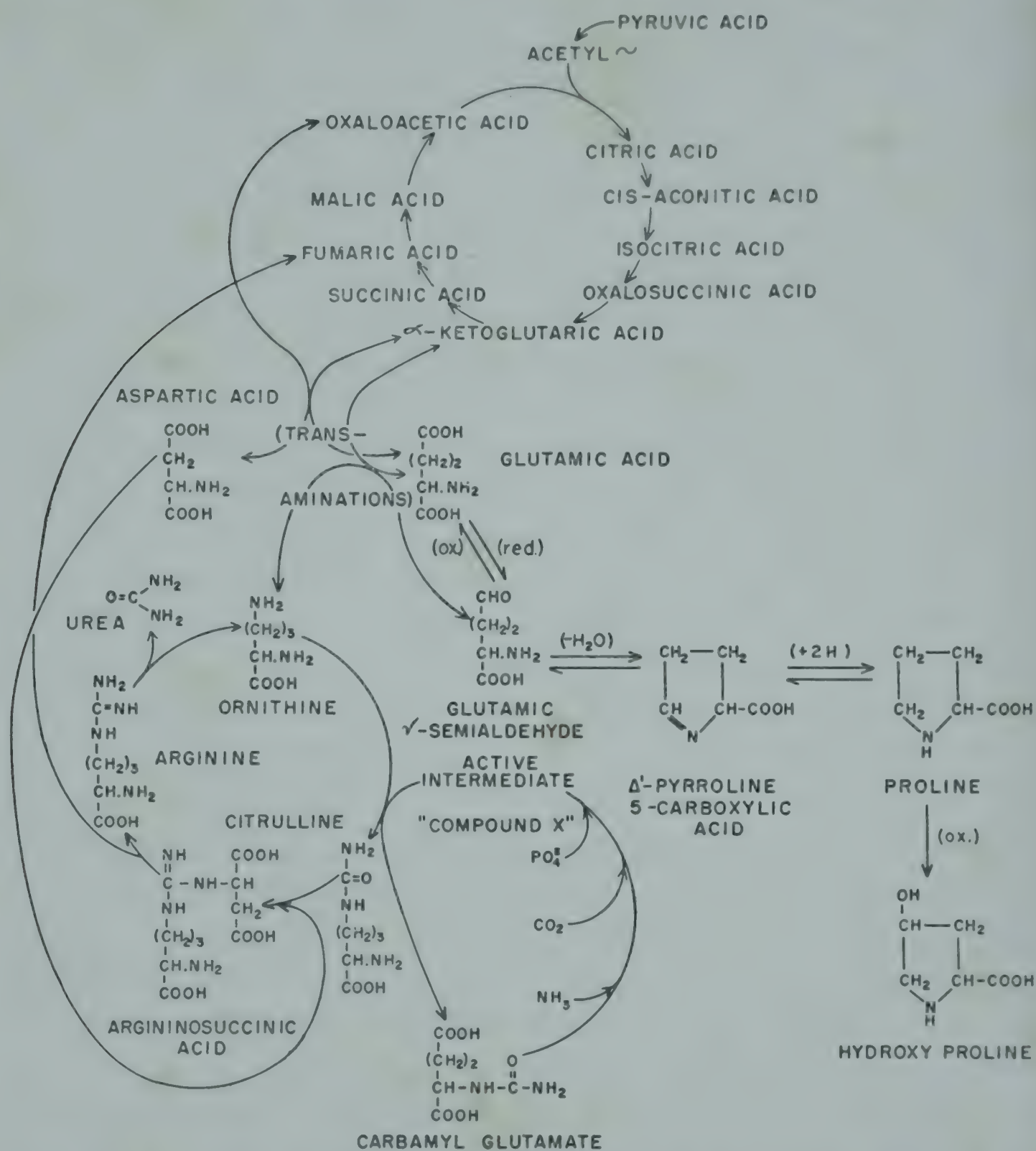


FIG. 4. The relation of glutamic acid to the citric acid and urea cycles and to the synthesis of proline and hydroxyproline by virtue of transaminations and reduction to glutamic-semialdehyde. (Based on the presentations of Ratner, Grisolia, and Stetten.)

inhibitors of respiration in general influence urea formation also. Malonate, which can inhibit urea formation without curtailing respiration very greatly, must on the other hand act by diminishing the

supply of oxaloacetate. Alpha-ketoglutarate inhibits in still another mode, by shifting the equilibrium in the transamination reaction toward the formation of glutamic rather than aspartic acid. Because liver cells are poorly permeable to both glutamic and aspartic acids, the formation of urea in liver slices is stimulated most when glutamine and ammonia are the exogenous sources, and presumably nitrogen is transported to the liver in those forms. Then, "once amino acids enter the cell, glutamic acid can act, under physiological conditions, as a funnel for transferring nitrogen from the general pool of amino acids into urea," for not only are there mechanisms that transfer nitrogen from glutamine and ammonia to form glutamic acid, but the numerous transaminase systems already discussed focus on glutamic acid because of the coupling with glutamic dehydrogenase, as suggested by Braunstein et al.

Three procedures were used by Busch and Baltrush for demonstrating the equilibration between glutamic acid and the citric acid cycle. By using fluoroacetate as an inhibitor, by studying the entry of  $C^{14}$  from various compounds into glutamate and  $\alpha$ -ketoglutarate, and by the degradation of glutamate, they succeeded in demonstrating that, at least in mammalian heart and kidney preparations, there is real equilibration between the citric acid cycle and glutamic acid. Fluoroacetate inhibits the labeling of glutamate and  $\alpha$ -ketoglutarate from acetate-1- $C^{14}$ , and both labeled acetate and pyruvate-2- $C^{14}$  strikingly label them, although labeled bicarbonate and labeled lactate do not. The flow of acetate into glutamate is far less in liver homogenates and mitochondrial preparations than in heart muscle homogenates, and the latter are considerably exceeded by kidney preparations. Transplantable tumor tissues showed a very much longer half-time for the utilization of acetate, and very little of the acetate got into glutamate.

Another aspect of the key position of glutamic acid was brought out in the contributions by Stetten and Vogel, in which the relationships between glutamic acid and proline, hydroxyproline, and ornithine in rats and in microorganisms were clarified (see Fig. 4). In rats proline is metabolized via glutamic acid. It can also be converted into ornithine, and the latter may be converted into proline



or glutamic acid. Hydroxyproline is derived in vivo from proline, and the hydroxyproline of the body, which is mainly found in collagen, has a very slow turnover with dietary hydroxyproline. The tissue hydroxyproline, in other words, is seemingly mainly derived from peptide or protein proline, and not from free hydroxyproline. The analysis of the pathways of interconversion has now revealed that glutamic  $\gamma$ -semialdehyde occupies an intermediate position in the conversion of glutamic acid to both proline and ornithine, for by transamination it may be converted to ornithine and through ring closure it may be converted to  $\Delta^1$ -pyrroline-5-carboxylic acid and thence to proline (Fig. 4). An alternative pathway from glutamic acid through  $\alpha$ -keto- $\delta$ -aminovaleric acid is not supported by isotope experiments with two kinds of N-labeled ornithine, for the  $\alpha$ -N atom goes mainly into proline and the  $\delta$ -N atom into glutamic acid. This result implies that the  $\delta$ -amino group goes mainly into a labile pool of nitrogen that includes glutamic and aspartic acids, and that the  $\alpha$ -amino group goes by another route, presumably that of the semialdehyde which spontaneously cyclizes, into proline and hydroxyproline. There does not seem to be any significant interchange of nitrogen between the amino groups of ornithine.

In *E. coli*, *Neurospora*, and the yeast *Torulopsis utilis* Vogel has demonstrated the existence of a similar pathway from glutamic acid to proline through glutamic  $\gamma$ -semialdehyde and  $\Delta^1$ -pyrroline-5-carboxylic acid. Mutant strains of *E. coli* have been found with blocks before glutamic acid, between glutamic acid and the semialdehyde, and between the semialdehyde and proline. The last of these excretes the glutamic  $\gamma$ -semialdehyde into the medium, where it enters into equilibrium spontaneously with its cyclization product, the latter appearing to predominate under physiological pH conditions. In *Neurospora* and *Torulopsis* the major path between glutamate and proline was also established by means of mutants, one of which has a block between proline and the semialdehyde, and another between glutamate and the semialdehyde. It therefore appears that the relationship of proline to glutamic acid is identical in mammals, exemplified by the rat, and in microorganisms. This did not hold true for ornithine synthesis, which has been shown, in



*E. coli*, to pass through N-acetylglutamate and N-acetyl glutamic  $\gamma$ -semialdehyde to N <sup>$\alpha$</sup> -acetylornithine and ornithine. It seems that in this case the reactivity of the  $\alpha$ -amino group of glutamic acid must be blocked by acetylation in order to permit the amination of the  $\gamma$ -carboxyl group. In *Neurospora* and *T. utilis*, however, the synthesis of ornithine does not occur through acetylated intermediates, according to evidence by Vogel from mutants. This contrast between *E. coli* and the two other microorganisms in regard to ornithine synthesis, and the similarity of the *Neurospora-Torulopsis* synthesis to that demonstrated by Stetten to occur in the rat led to a lively exchange of views between those participants in the symposium who, like Vogel, upheld the merits of *Neurospora* as a biochemical model of mammalian metabolism, and those who thought that something worthwhile might still come out of *E. coli*. Vogel bolstered his argument by pointing out that in *E. coli* there are other links between proline and ornithine than those involving glutamate, and that exogenous ornithine can be converted into proline by a minor path; whereas in *Neurospora* and *Torulopsis*, although exogenous ornithine may yield proline, endogenous ornithine is not effectively utilized for that function. Vogel hence suggested that the paths of proline and ornithine synthesis are "physically separated" in *Neurospora* and *Torulopsis*, and likewise in mammals, with a "restrictive channeling of metabolites" as a result. In some way the unfavorable equilibrium in the transamination leading to ornithine, so far on the side of the semialdehyde, is counteracted in these forms; whereas in *E. coli*, which lacks this mechanism, the synthesis of ornithine from glutamic  $\gamma$ -semialdehyde is inadequate, and the acetylated intermediates must be relied upon. From an evolutionary standpoint it is of course inconceivable that *Neurospora* and yeast, any more than bacteria, lie on the direct path to the higher animals. Either these similarities represent an evolution of parallel but independent mechanisms, or the bacteria exemplified by *E. coli* are the divergent forms. It is hard to draw conclusions when so few types of organisms have been studied biochemically. Perhaps the glutamate-ornithine-proline metabolism of the protozoa and lower metazoans would shed some light on so interesting a problem.



## HISTIDINE, LEUCINE, ISOLEUCINE, VALINE, AND LYSINE

*Histidine* (see Fig. 5)

The biosynthesis of histidine (Fig. 5, top) has been worked out mainly, according to Ames, from the effects of blocking steps in the normal synthetic pathway. Imidazole pyruvic acid was first shown to be a normal precursor in *Lactobacillus arabinosus* and *Streptococcus faecalis*, and pyridoxal phosphate is a necessary co-factor in the conversion to histidine. In various yeasts (and also in slices of human liver, according to Coon in the discussion) formate C<sup>14</sup> was found to be incorporated into the imidazole ring at position 2; and glycine, which enters the imidazole ring of purines, could not do so in the synthesis of histidine. This fact eliminated any direct participation of the purines in histidine synthesis. By other tracer experiments it was demonstrated that the main carbon chain of histidine is derived directly from glucose rather than from glutamic acid or acetate. The mutants of *Neurospora crassa* have, however, been most revealing in eliciting the details of the synthesis of histidine. These mutants, which fall into five groups, all require histidine very specifically, no other imidazole compounds being able to serve as substitutes for histidine. None of the mutants accumulated substances that stimulated the growth of other mutants, but three were found that accumulated imidazoles, and by using double mutants a sequence of steps was worked out, leading from imidazole glycerol to imidazole acetol and thence to L-histidinol. An imidazole glycerol obtained from *Neurospora* turned out to be the D-erythro form, which can be synthesized either from D-arabinose or D-ribose, and the synthesis of histidine was thus carried back to the pentoses. The mystery of the inactivity of the supposed intermediate compounds when supplied exogenously led to a suspicion that the actual intermediates might be more complex molecules to which the *Neurospora* mycelium is impermeable. A search then disclosed the accumulation of the three corresponding phosphate esters in respective mutants, and as expected they were found to be unable to replace histidine when supplied in the medium because the mycelium is not permeable to them. The two enzymes responsible for the intercon-

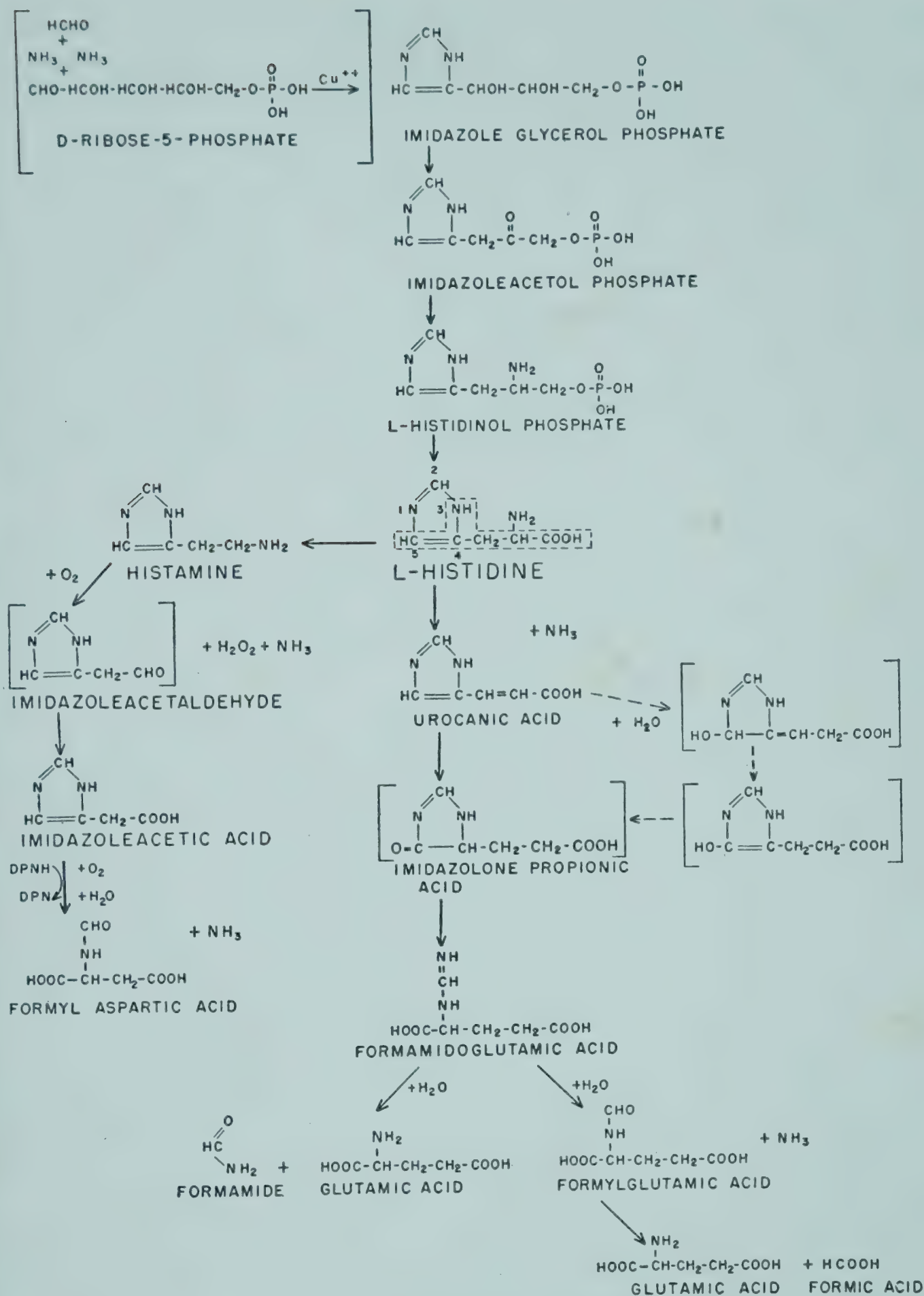


FIG. 5. The biosynthesis and degradation of histidine, based on the presentations of Ames, Tabor, Magasanik, Waelsch, and Hayaishi. The degradation of histamine to formyl aspartic acid is based on Hayaishi's studies of *Pseudomonas fluorescens*. The shunt to the right between urocanic acid and imidazolone propionic acid represents Waelsch's postulated intermediates. The forking pathways below formamidoglutamic acid represent the degradative paths in *Aerobacter aerogenes* and *Clostridium tetanomorphum*, to the left; and in *Pseudomonas fluorescens*, to the right.



versions have also been obtained, for the first step an imidazole glycerol phosphate dehydrase, presumably metal-dependent; for the second step an imidazole acetol phosphate transaminase that transfers the amino group from glutamate to the phosphate ester. This transamination, which requires pyridoxal phosphate as a cofactor, is quite unusual in that the recipient has a phosphate ester group instead of a carboxyl group. In *E. coli* and other bacteria a DPN-requiring enzyme that oxidizes L-histidinol to histidine has been found (Adams), but in *Neurospora* there is no indication that histidinol is itself a precursor of histidine; probably the phosphate group is taken off at the same time as the histidinol is oxidized to histidine (Fig. 5).

The degradation of histidine (Fig. 5) was discussed by Tabor, who pointed out that nearly all the histidine administered to animals is eventually degraded to  $\text{CO}_2$ . Isotope experiments with histidine-adapted *Pseudomonas fluorescens* cells have established the degradation products as L-glutamic acid, formic acid, and ammonia. The primary step is a simple deamination which converts histidine into urocanic acid, and glutamic acid is produced from the portion of the histidine molecule surrounded by dotted lines in Fig. 5. The released formate then comes from the carbon group into which formate is incorporated during histidine synthesis. Histidase (histidine deaminase) is found in mammalian liver and in a number of bacteria. Urocanase then degrades urocanic acid to an intermediate compound, now identified as formamido-L-glutamic acid (called alternatively  $\alpha$ -formamidino-glutaric acid), and studied by Tabor, by Magasanik and Bowser, and by Waelsch and Miller. Waelsch postulated a very interesting mechanism for the steps in the conversion of urocanic acid to the formamidoglutamic acid (see Fig. 5, middle right). His suggestion was that of an addition of water (1:4) followed by a 3-carbon tautomerization leading to the enol form of imidazolone-propionic acid, which in its keto form is postulated to be the direct precursor of the formamidoglutamic acid. This hypothesis led Knox to tell of finding an enol-keto tautomerase of phenylpyruvic acid during his studies of tyrosine metabolism.

In liver preparations the degradation stops with the formation



of formamidoglutamic acid; but in *Pseudomonas* extracts this intermediate compound is in turn degraded to formylglutamic acid and ammonia, and the formylglutamic acid is finally broken down into glutamic acid and formate (Fig. 5, bottom right). Formylisoglutamine, once supposed to be an intermediate, has now been eliminated. In *Aerobacter aerogenes*, on the other hand, formamidoglutamic acid is converted in histidine-adapted cells to glutamic acid and formamide, according to Magasanik (Fig. 5, bottom left). The glutamate is assimilated, but the formamide is not further attacked. Formylglutamic acid is not an intermediate in these steps in *Aerobacter*.

Particularly interesting is the relationship of histidine degradation to folic acid. Rats suffering from a folic acid deficiency excrete the histidine degradation product, formamidoglutamic acid, in the urine, although normal rats do not. A further tie between histidine and folic acid function lies in the utilization of the formate obtained from C<sub>2</sub> of the imidazole ring of histidine by serine, imidazole carboxamide, and other formate acceptors. Stimulated by a recent Japanese report that the enzymatic step from histidine to urocanic acid is folic-acid-dependent, Waelsch speculates that the utilization of one-carbon units from histidine may occur early in the degradation of histidine and represent a different pathway that starts with the activation of carbon-2 of histidine and involves an unknown intermediate that serves as donor of the one-carbon unit, and may at the same time be a precursor of urocanic acid.

Another pathway of histidine degradation, one of much pharmacological interest, is the decarboxylation that results in the formation of histamine (Fig. 5, middle left). The enzyme involved, histidine decarboxylase, has been chiefly studied in various microorganisms. In animal tissues its activity is very low. Histamine is in turn degraded by histaminase, or diamine oxidase, for this enzyme also works on other substrates. It presumably produces imidazole-acetaldehyde, which has not been isolated, for the product is converted to imidazoleacetic acid by either xanthine oxidase or aldehyde dehydrogenase with DPN. Hayaishi discussed in particular the fate of the imidazoleacetic acid thus produced. In *Pseudomonas fluorescens* extracts imidazoleacetic acid is converted by means of an



adaptive enzyme and DPNH into formylaspartic acid and ammonia (see Fig. 5, left). The organisms adapted to imidazoleacetic acid can oxidize formylaspartate as well as aspartate, without a lag period, and both these compounds are thus implicated as intermediates of imidazoleacetic acid metabolism.

*The Branched-Chain Amino Acids* (see Fig. 6)

From a study of mutants of *E. coli* and *Neurospora*, reported by Adelberg and by Umbarger, and from tracer studies carried out in the yeast *Torulopsis utilis* and reported by Strassman and Weinhouse, the pathways in the biosynthesis of isoleucine, valine, and leucine have been considerably extended. The remarkable double requirements of certain mutants for valine and isoleucine, once thought to be due to the inhibition of valine synthesis by the accumulation of some isoleucine precursor, have been shown to be brought about by a striking parallel transformation of the corresponding dihydroxy acids to the keto acids and of the keto acids to the amino acids by two enzymes, one of which dehydrates either dihydroxy acid while the other transaminates to either keto acid. Although threonine donates four carbon atoms to the dihydroxy acid precursor of isoleucine, it does not, as was formerly thought, contribute to valine; but the dihydroxy acid precursor of the latter is derived from pyruvic acid. Adelberg's isotopic competition experiments with a mutant that accumulates the dihydroxy acids showed that threonine carbons 1 and 2 become isoleucine carbons 1 and 2, but threonine carbons 3 and 4 separate from carbons 1 and 2 and appear as carbons 5 and 6 of isoleucine. Adelberg proposes (Fig. 6, 1) that threonine, which is known to be converted to  $\alpha$ -ketobutyric acid before being used for isoleucine synthesis, undergoes an aldol condensation with pyruvic acid to form a 7-carbon keto acid. This in turn, by enolization and hydration, might form a pinacol, which by rearrangement and decarboxylation would give the carbon skeleton of isoleucine in the form of a  $\beta$ -keto acid. Further enolization and hydration might then yield a trihydroxy acid, whence the dihydroxy acid might be derived. A similar mechanism, starting with a condensation of two molecules of pyruvic acid, might be invoked for the origin of the dihydroxy

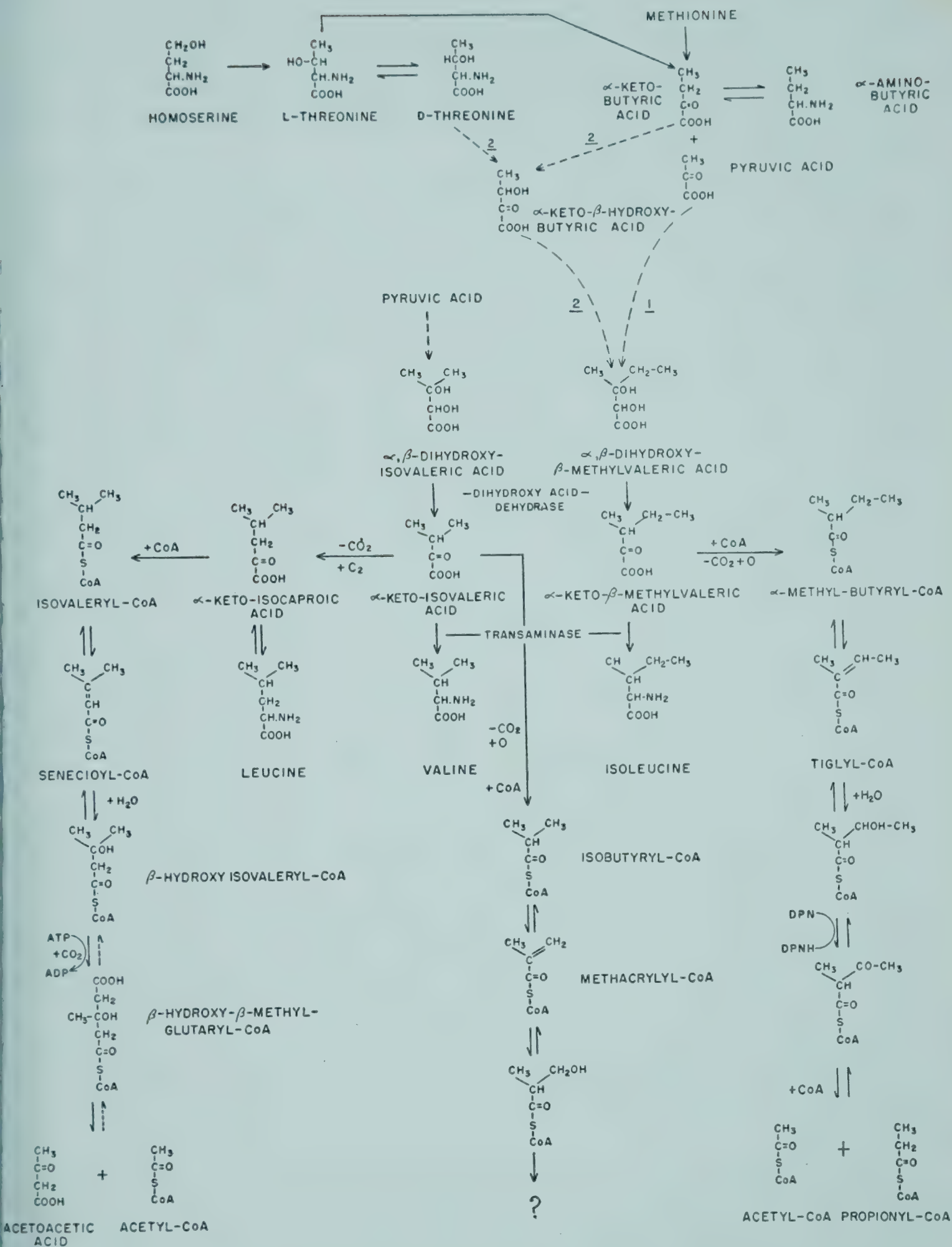


FIG. 6. The biosynthesis and degradation of the branched-chain amino acids, according to Adelberg, Coon, and Umbarger.



acid precursor of valine. Leucine, according to the isotopic competition experiments of Abelson, comes from its keto acid analogue, which in turn is formed from the keto acid analogue of valine. The nature of this reaction is still problematical, but the final step is no doubt a leucine transamination.

Umbarger discussed the earlier steps in the synthetic pathway of isoleucine, namely, those which lie between threonine and the dihydroxy acid precursor. Evidence was derived from *E. coli* mutants whose isoleucine requirement could be supplied respectively by (1) L-threonine, (2) D-threonine, or (3) L-threonine and either D-threonine or  $\alpha$ -aminobutyric acid. In the strain with the double block the isoleucine requirement was unchanged, although the strain when supplied with isoleucine responded more efficiently to L-threonine than the singly blocked L-threonine-requiring strain. This was taken as evidence that in the latter strain a part—maybe one-half—of the L-threonine is diverted into isoleucine synthesis; and from this and other evidence Umbarger concluded that L-threonine is a direct precursor of isoleucine. Davis, in the discussion, disagreed with this interpretation, and argued from other evidence, also derived from *E. coli* mutants, that L-threonine lies to the side of the main pathway of isoleucine synthesis. Vogel in turn supported Umbarger's contention, and listeners were clearly left with the impression that the issue is not by any means settled. Another puzzle is the discovery that isoleucine-requiring mutants which are unable to utilize L-threonine and homoserine, its precursor, nevertheless possess a threonine racemase which, if D-threonine is on the direct path, should enable them to synthesize isoleucine unless some subsidiary hypothesis about the low activity of the enzyme is adduced. And still unexplained is why these same mutants can utilize  $\alpha$ -ketobutyric acid yet not L-threonine, although the cells demonstrably contain threonine deaminase.

In Umbarger's scheme, the pathway from  $\alpha$ -ketobutyrate to isoleucine (Fig. 6, 2) leads through  $\alpha$ -keto- $\beta$ -hydroxybutyric acid, the keto analogue of threonine. As yet no evidence exists to show how  $\alpha$ -ketobutyric acid can be converted into this compound; nor has there been found any transaminase or dehydrogenase that will



convert D-threonine, which is only utilized under highly aerobic conditions, into its keto-analogue. The further steps from the keto analogue of D-threonine to  $\alpha,\beta$ -dihydroxy- $\beta$ -methylvaleric acid remain uncertain. The two final steps are those already considered by Adelberg, and represent the steps from the dihydroxy to the keto acid analogue that parallel those in the synthesis of valine and are catalyzed by common enzymes.

The isotope studies on the yeast *Torulopsis* indicated that whereas the carbon atoms of acetate are not readily incorporated into valine, those of lactate are freely utilized. Strassman and Weinhouse pointed out that the equal labeling of carbons 2 and 3 of valine by the  $\alpha$ -carbon of lactate implies that two  $\alpha$ -carbons of lactate must undergo coupling. These workers have consequently postulated a condensation and migration mechanism almost like that proposed by Adelberg. The observed distributions of the  $C^{14}$  tracer atoms in isoleucine synthesis agreed very well with those expected, but although the scheme is rather simpler than Adelberg's it nevertheless fails to reckon with the participation of the dihydroxy analogues of valine and isoleucine which have been proved to exist in *E. coli* and *Neurospora* and to be normal precursors of valine and isoleucine.

The degradation of the branched-chain amino acids (see Fig. 6) is apparently never by means of demethylation at any stage, but occurs by deamination followed by decarboxylation of the keto acid analogues. The products would be branched-chain fatty acids. Thus, leucine, which is the most strongly ketogenic of the three amino acids in this group, first goes back to  $\alpha$ -ketoisocaproic acid in its degradation. Coon and his coworkers have provided parallel schemes for the degradation of isoleucine and valine as well as leucine. They point out that in the degradation of leucine there are two steps of particular interest because they differ from any in straight-chain fatty acid metabolism. These are (1) carbon dioxide fixation, and (2) cleavage of the chain without prior formation of a  $\beta$ -keto acid. Having demonstrated that thiol esters are involved, Coon and his group have proceeded to postulate that in the case of leucine (Fig. 6, lower left) the keto-acid analogue,  $\alpha$ -ketoisocaproic acid, first combines with Coenzyme A. Next there is an oxidation



to an unsaturated chain; and this is followed by the addition of water to yield  $\beta$ -hydroxyisovaleryl-CoA, in a step actually demonstrated in heart preparations. In a reaction requiring ATP,  $\text{CO}_2$  is then fixed as a carboxyl group at the end of the chain, which finally splits into acetoacetate and acetyl-CoA. The intermediate compound in the last step has been identified as  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA. Some of the branched-chain intermediates may be used in cholesterol synthesis, for which such forms are required; and if so, that use represents the only known metabolic function of leucine outside of protein synthesis.

Isoleucine and its derivative,  $\alpha$ -methylbutyric acid, are only weakly ketogenic and are definitely glycogenic. The reaction scheme proposed by Coon is quite similar to that for leucine, except that a DPN-requiring dehydrogenase reaction replaces the fixation of  $\text{CO}_2$ , and that the final step is a CoA cleavage to acetyl-CoA and propionyl-CoA. Valine is similarly assumed to pass through the keto acid,  $\alpha$ -ketoisovalerate. This is oxidatively decarboxylated to isobutyrate and converted to the thiol ester, which in turn is oxidized to the unsaturated form (methacrylyl-CoA). This was found to undergo hydration in a heart enzyme system to a new thiol ester (see Fig. 6, lower center), the fate of which remains at present unknown.

### *Lysine* (Fig. 7)

Lysine metabolism shows so much variation from species to species that it is difficult to construct any general pattern. E. Work has contrasted the differing modes of lysine degradation in the rat, in *Neurospora*, and in *E. coli* and other bacteria, and its synthesis in all of these except the rat.

In animals lysine is a required amino acid, so that the steps leading to its synthesis are effectively blocked. Lysine does not take part in the general transaminations of the other amino acids, and consequently does not contribute to the metabolic pool of nitrogen. Its oxidative deamination is also very slow, although in this case, as in transaminations,  $\epsilon$ -N-substituted derivatives show some activity. In vivo the degradation of lysine in the rat begins with deamination at the  $\alpha$ -carbon site. It was formerly thought, from the appearance



of  $\alpha$ -aminoadipic acid [ $\text{COOH} \cdot (\text{CH}_2)_3 \cdot \text{CHNH}_2 \cdot \text{COOH}$ ] as a degradation product that the  $\epsilon$ -carbon was first deaminated. The discovery in plants of the cyclic product of lysine, pipecolic acid, led to a reconsideration of the supposed pathway. Pipecolic acid, later found also to occur in the rat, is apparently formed by deamination of the  $\alpha$ -carbon, followed by ring closure. This ring structure is capable of reopening in such a way as to transfer the amino group from the  $\epsilon$ -carbon to the  $\alpha$ -carbon, and to make  $\alpha$ -aminoadipic acid, probably by way of a semialdehyde.  $\alpha$ -Aminoadipic acid can now be deaminated to the keto acid, which is converted into glutaric acid and thence into  $\alpha$ -ketoglutarate. This degradative pathway is irreversible and the  $\alpha$ -carbon is unable to undergo amination except by transfer from the  $\epsilon$ -carbon. The failure in this case of the oxidative and transaminative mechanisms that interconvert so many D- and L-amino acids is illustrated by the fact that D-lysine is unable to replace L-lysine in the diet of the rat. It seems probable, as Work suggests, that the rapid ring closure to which lysine is subject prevents reamination at the  $\alpha$ -carbon.

In *Neurospora*, according to the work of Schweet and co-workers,  $\alpha$ -aminoadipic acid is a precursor of lysine, by way of the semialdehyde, which can also be formed from  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid [ $\text{CH}_2\text{OH} \cdot (\text{CH}_2)_3 \cdot \text{CHNH}_2 \cdot \text{COOH}$ ]; and pipecolic acid is not a precursor. Schweet and his coworkers have presented evidence that in *Neurospora*  $\alpha$ -keto- $\epsilon$ -aminocaproic acid is formed from L-lysine by L-amino acid oxidase, and exists in solution in its cyclized form,  $\Delta^1$ -dehydropipecolic acid. It can be converted back to pipecolic acid and lysine in vivo. These workers regard the cyclic form as the intermediate compound between lysine and pipecolic acid. *Neurospora*, unlike the rat, can utilize D-lysine, so that a transamination system may exist. The hypothetical semialdehyde [ $\text{CHO} \cdot (\text{CH}_2)_3 \cdot \text{CHNH}_2 \cdot \text{COOH}$ ] may serve as the recipient of the second amino group. Work has suggested a composite scheme to bring out the differences and similarities in lysine metabolism in *Neurospora* and the rat (Fig. 7). The main cause of differences, she thinks, is due to the behavior of the semialdehyde, which in *Neurospora* is aminated to lysine and in the rat can only be oxidized to  $\alpha$ -aminoadipic





reamination occurs would possibly tend to prevent ring closure and so to favor the removal or addition of the other amino group, as in ornithine biosynthesis. The hypothesis is supported by some evidence. An  $\epsilon$ -N-acylated lysine derivative has actually been obtained in *Neurospora* (Schweet), and in the rat dietary lysine can be replaced by the  $\epsilon$ -N-acetylated derivatives, which are labile in the rat, but cannot be replaced by the  $\alpha$ -N-acetyl derivatives.

In *E. coli*, and most other bacteria (except the cocci), and in the blue-green algae as well, the previously mentioned precursors of lysine in *Neurospora* are ineffective, and instead diaminopimelic acid  $[\text{COOH} \cdot \text{CHNH}_2 \cdot (\text{CH}_2)_3 \cdot \text{CHNH}_2 \cdot \text{COOH}]$  is used. Another difference is that in *E. coli* the carboxyl of lysine is derived from  $\text{CO}_2$ , whereas in *Neurospora* and yeast it is not. Davis found a mutant in *E. coli* that had an absolute requirement for diaminopimelic acid combined with a relative requirement for lysine, and Work and her coworkers found in the same organism and in *Aerobacter aerogenes* an enzyme that is capable of decarboxylating diaminopimelic acid to yield lysine. This enzyme, which possesses optimal activity at pH 7, differs from other known bacterial amino acid decarboxylases, which have optima in the acid range and presumably merely protect against the adverse effects of an acid environment. The importance of this diaminopimelic decarboxylase enzyme has been established by showing that it is absent in each of three *E. coli* mutants with an absolute lysine requirement, but is present in the aforementioned mutant with a requirement for diaminopimelic acid. Moreover, the three former mutants each accumulate diaminopimelic acid in the medium. The evidence that diaminopimelic acid is a normal precursor of lysine in *E. coli*, and presumably in other bacteria, is thus conclusive. Diaminopimelic decarboxylase exhibits a very sharp specificity. It attacks the carboxyl in the D-configuration, whereas lysine decarboxylase attacks only that in the L-configuration. Diaminopimelic decarboxylase requires pyridoxal phosphate as a cofactor, and shows some adaptive increase when both lysine and pyridoxine deficiencies exist. The enzyme is apparently more widely distributed among bacteria than its substrate, but a few instances were found in which the opposite relation was true (DAP but no enzyme).



From the growth requirements of other mutant strains, both threonine and aspartic acid seem to be involved in the synthesis of diaminopimelic acid. These relationships can be explained by assuming that threonine and diaminopimelic acid have a common precursor. Since the synthesis of threonine from aspartic acid by way of homoserine is established for these bacteria, and since homoserine is not a lysine precursor (Abelson), the common precursor of threonine and diaminopimelic acid is perhaps one of the postulated intermediates in threonine synthesis,  $\beta$ -aspartyl phosphate or aspartic semialdehyde (see below). There is some evidence for an alternative route of lysine synthesis which does not involve diaminopimelic acid, but is inhibited by that compound.

Bacteria (*E. coli*, *Clostridium*) convert lysine to butyric acid, acetic acid, and ammonia, according to Thressa Stadtman, by a process that involves no overall oxidation or reduction.  $C^{14}$  tracer studies revealed that apparently a two-carbon piece can be split from either end of the lysine molecule. The intermediary role of  $\alpha$ -aminoadipic acid seems unlikely in this case, but that of pipecolic acid has not been ruled out.

In concluding in regard to lysine metabolism, it may be said that the bacteria seem to have found yet another way of overcoming the tendency of the six-carbon amino acid to close into a ring-form. Instead of stabilizing the amino groups by acylation, as molds and mammals do, the bacteria synthesize a seven-carbon compound and then decarboxylate it.

#### THE INTERRELATIONSHIPS OF METHIONINE, CYSTEINE, AND THREONINE

Stekol has presented a comprehensive scheme of the general interrelationships in the synthesis of methionine, cysteine, and threonine (Fig. 8). His own review dealt in particular with the synthesis of cysteine and of methionine, with methyl and thiomethyl transfers, and with the genesis of the methyl group from formate and other one-carbon units. The synthesis of threonine was reviewed by Black.

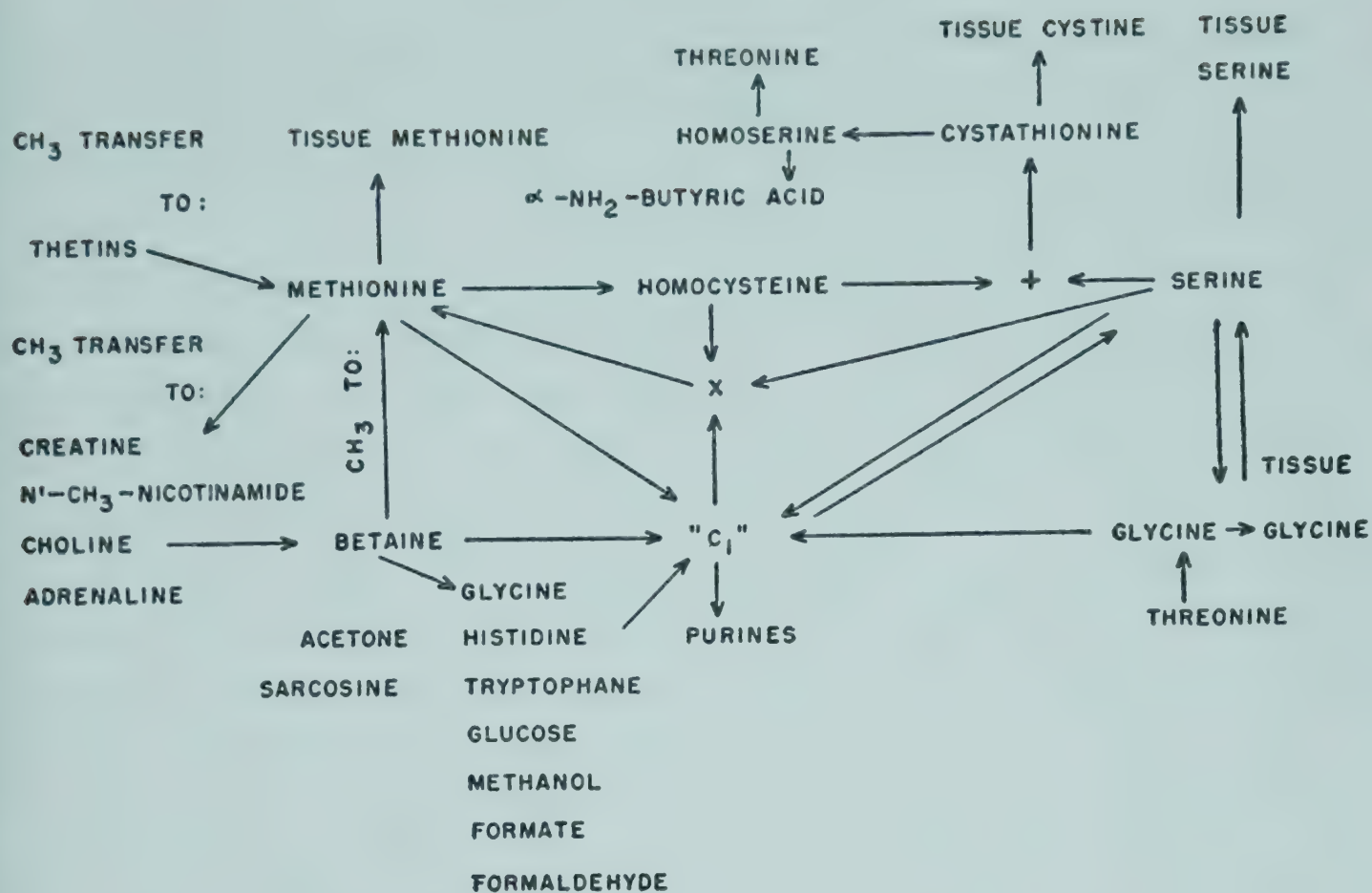


FIG. 8. General interrelationships in the synthesis of methionine, cystine, and threonine. (From Stekol.)

### *The Synthesis of Cysteine ( $\text{SH} \cdot \text{CH}_2 \cdot \text{CHNH}_2 \cdot \text{COOH}$ )*

It is now well recognized that in one of the major routes of the synthesis of cysteine a thioether, cystathionine,  $[\text{COOH} \cdot \text{CHNH}_2 \cdot (\text{CH}_2)_2 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CHNH}_2 \cdot \text{COOH}]$ , is an intermediate. It is formed from the condensation of homocysteine and serine. Of the two active diastereoisomers, L-cystathionine is cleaved to cysteine and homoserine, L-allocystathionine to homocysteine and serine. The enzyme that cleaves cystathionine is stable at 50° C., whereas the enzyme that condenses homocysteine and serine is not. This fortunate difference in properties enabled the two enzymes to be studied separately, and it was found that thionase, the cystathionine-cleaving enzyme, splits a number of other thioethers, such as lanthionine and djenkolic acid. In 1950 Russian workers demonstrated that pyridoxal phosphate is essential to the synthesis of cysteine from homocysteine and serine. Binkley et al. have discovered that it is the coenzyme for the condensing enzyme as well as the thionase. The function of the coenzyme is interpretable on the assumption that it forms a Schiff base with serine, followed by dehydration of the attached serine



to aminoacrylic acid. The long conjugated system in the Schiff base would stabilize it, and the double bond would permit homocysteine to be added across the double bond so as to give cystathionine—a suggestion made by Metzler and Snell.

In animals a considerable proportion of dietary methionine is used for the synthesis of cysteine and cystine. In view of the fact that in many parts of the world human diets are low in methionine, the sparing action of ample cysteine assumes great practical significance. On the other hand, a diet low in cystine, at least for the rat, cannot be alleviated by an oversupply of methionine. Part of this effect may be due to the fact that the carbon skeleton of cysteine comes from serine and glycine, and not from methionine. The adequacy of many enzyme systems is involved, over and above those that directly transfer the sulfur moiety from methionine to form cysteine. Thus a folic acid deficiency may be of great importance in cysteine synthesis because of its interference with the incorporation of active formate into serine (see below).

Among compounds that can donate sulfur to form cysteine are the artificial thioethers, lanthionine and homolanthionine, produced by alkali-treatment of wool; ethionine, which acts analogously to methionine; and inorganic sulfur ( $\text{Na}_2\text{SO}_4$ ). Sulfate, although most effectively utilized by ruminants through the agency of their rumen bacteria, can apparently be reduced and utilized by rabbits and chickens. Following its reduction to hydrogen sulfide, the sulfur might in these animals be incorporated into cysteine by a reversal of the action of cysteine dehydrase (desulfhydrase). Aminoacrylic acid [ $\text{CH}_2 = \text{CNH}_2 \cdot \text{COOH}$ ], needed as a recipient of the  $-\text{SH}$  group, could be produced from serine by serine dehydrase.

In the discussion, Horowitz brought out some interesting aspects of the pathway from inorganic sulfate through cysteine to methionine in *Neurospora*. Of approximately 100 independently obtained blocks in the synthesis of methionine in this organism, roughly half lie in the steps prior to the formation of cysteine. From the study of these blocks, Horowitz thinks that the principal pathway for the incorporation of inorganic sulfur into cysteine and methionine in *Neurospora* is an inorganic route, proceeding in order through



the steps sulfate : sulfite : thiosulfate : elemental sulfur : sulfide : cysteine. There are mutants which block each of these steps except the last two, but all which are blocked between thiosulfate and cysteine are unable to reduce sulfur. Cysteic acid and cysteine sulfinic acid perhaps feed into this sequence at the step leading to thiosulfate, for mutants which block the conversion of sulfite to thiosulfate also prevent the utilization of cysteate or cysteine sulfinic acid. There is accordingly no evidence at present for a separate organic pathway for the reduction of sulfur in *Neurospora*. Singer, however, questions the conversion of cysteine sulfinic acid and cysteic acid to sulfite, and regards the evidence as implying instead only the existence of a pathway from inorganic sulfur (viz., sulfite) to organic sulfur ( $\beta$ -sulfinylpyruvate and cysteine).

*The Synthesis of Methionine* [ $\text{CH}_3 \cdot \text{S} \cdot (\text{CH}_2)_2 \cdot \text{CHNH}_2 \cdot \text{COOH}$ ]

By transmethylation from any compound with a methyl group attached to an onium pole, homocysteine can be converted into methionine. These transmethylations occur from a number of donors: betaine, methionine sulfoxide, methylthetin, methylpropiothetin, methionine methylsulfonium, and methionine ethylsulfonium. [In the discussion, Du Vigneaud cautioned against the use of the terms "labile methyl compound" and "methyl donor" in any except a nutritional sense, since otherwise, with the discovery of new intermediate compounds, it might repeatedly be necessary to reclassify the list of donors, just as, for example, choline had to be removed from the list when betaine was found to be an intermediate.] The transfers do not require energy-rich phosphate, since the onium structure is already at the "energy-rich" level. Methyl methionine sulfonium, which occurs naturally, will support the growth of rats and of certain bacteria, at about 80 to 90 per cent of the efficiency of methionine itself. Stekol finds that the transfer of the methyl group from the thetins, among which methyl methionine sulfonium may be included, is not a simple, direct process; and he proposes that betaine and the thetins form methyl esters, such as dimethylglycine methyl ester [ $(\text{CH}_3)_2 \cdot \text{N} \cdot \text{CH}_2 \cdot \text{COO} \cdot \text{CH}_3$ ] arising from betaine, and that these methyl esters then donate the methyl group to homocysteine. The



transmethylations from methionine to form choline or creatine, and those in the opposite direction, to form methionine from choline or betaine, are not affected by a deficiency of vitamin B<sub>12</sub>. On the other hand, a folic acid deficiency has an inhibitory effect.

According to Stekol, the methyl group of methionine, like those of choline, creatine, and thymine, may also be synthesized *de novo* from active one-carbon units, provided vitamin B<sub>12</sub> and folic acid are adequate in amount. Not only do deficiencies of vitamin B<sub>12</sub> and folic acid inhibit the synthesis of the methyl groups of methionine, choline, and creatine from the  $\alpha$ -carbon of glycine, but pyridoxine and pantothenic acid deficiencies do so likewise. Thus, in the living rat, at least four cofactors affect the reactions from glycine to methionine.

Additional light was thrown on the problem of the origin of new methyl groups by Sprinson. By a series of experiments in which the  $\beta$ -carbon of serine was labeled with C<sup>14</sup> and attached deuterium, it was shown that the new methyl groups which are transferred to methionine, choline, thymine, and the purines are formed from the hydroxymethyl group of serine without any rupture of H-C bonds, unless there might be some selective cleavage of carbon-protium as against carbon-deuterium bonds. Additional experiments seem to rule out that possibility. The involvement of folic acid or its derivatives in the utilization of one-carbon units has led Sprinson to postulate, identically with Welch and Nichol, that a hydroxymethylated coenzyme might be the active unit in the genesis of new methyl groups from serine and glycine. This new suggestion is analogous to the earlier proposal that a formylated coenzyme might be the "active formate" involved in the utilization of administered formate, or of the one-carbon unit derived from histidine or tryptophan in the synthesis of purines. There is a preferential movement of the active one-carbon unit from serine into methionine, etc., and of formate or the one-carbon unit from histidine or tryptophan into the purines. To allow for this, and at the same time for the interconversion of the two biologically active forms of folic acid, Sprinson proposes the scheme shown in Fig. 9. The position of glycine







give rise to hydroxymethylhomocysteine. But it seems equally likely that the reduction might take place at the time of transfer, so as to yield methionine directly.

Especially interesting in relation to the synthesis of methionine is the discovery of a mutant of *Aerobacter aerogenes* that transfers to  $\alpha$ -aminobutyric acid the combined thiomethyl group from thiomethyladenosine. In this case methionine is synthesized by a direct reversal of the path of methionine degradation in yeast. In animal tissues methyl mercaptan [ $\text{CH}_3\cdot\text{SH}$ ] is formed from methionine, and in vivo the carbon atom of methyl mercaptan can be recovered in methionine. Since in yeast a similar utilization of methyl mercaptan leads to the transfer of the thiomethyl group from methionine to adenosine, it seems quite possible that in animals too methionine may transfer the thiomethyl group to adenosine, and a reverse transfer to the degradation product,  $\alpha$ -aminobutyric acid, may regenerate methionine.

A different mechanism was proposed by Cantoni. He pointed out that in spite of the very great importance of the metabolic functions assigned to homocysteine, there is still no evidence that it occurs as a free amino acid, although the homocysteinyl radical is present in both methionine and cystathionine. The discovery that in transmethylation reactions methionine itself does not act as a methyl donor, but is first enzymatically activated by conversion to S-adenosylmethionine, led Cantoni to investigate the possibility that S-adenosylhomocysteine might be the primary product of transmethylations from methionine. He was able to identify S-adenosylhomocysteine chromatographically as a product in the course of cysteine synthesis from S-adenosylmethionine and to show that it is identical to synthetic adenosylhomocysteine. Cantoni, in a series of interesting speculations, went on to suggest that this new compound may act as an acceptor of the methyl group from the thetins, betaine, and other onium compounds and so regenerate adenosylmethionine; that it might serve as the primary acceptor of the one-carbon units derived from formate and glycine; that it might be involved in cysteine synthesis by directly producing cystathionine; and that it might undergo hydrolysis or phosphorolysis to yield free homocysteine, if such



is found to occur naturally. If this mechanism is very general, the transfer of the thiomethyl group from methionine or thiomethyl adenosine, discussed above, cannot be of major quantitative significance.

Much future work will be needed to establish the relative significance of the five paths for the synthesis of methionine which have been discussed here, namely: by the direct transfer of the methyl group respectively from (1) N-onium compounds such as betaine, or from (2) S-onium compounds such as the thetins; (3) by transmethylation; (4) by reversal of the synthesis of cysteine from methionine, perhaps through the formation of adenosylmethionine from adenosylhomocysteine; and (5) by genesis of the methyl group of methionine de novo from one-carbon units. At present there is only a little evidence bearing on this, such as Stekol's report that an addition to the animal's diet of choline failed to suppress the genesis of methyl groups from formate, glycine, or serine; and that therefore this route, for the transfer of methyl groups from choline through betaine to methionine, is relatively inefficient. The great rapidity and extent of other transmethylation indicates that these constitute major reactions in the synthesis and degradation of methionine.

### *The Degradation of the Sulfur Amino Acids*

Various aspects of the pathways whereby the sulfur-containing amino acids are degraded were considered by Singer.  $\alpha$ -Ketobutyrate may be formed from homocysteine either indirectly by the deamination of the homoserine formed from the splitting of cystathionine, as in *Neurospora*, or directly by removal of  $\text{NH}_3$  and  $\text{H}_2\text{S}$ , as in *Proteus morganii*.

Kallio presented in the symposium some analyses of methionine degradation in several strains of *Pseudomonas*. In this bacterium the end-products of the oxidation of methionine are ammonia and dimethyl disulfide ( $\text{CH}_3\cdot\text{S}\cdot\text{S}\cdot\text{CH}_3$ ). If arsenite is added the respiration is considerably inhibited, and an amino acid, which has been identified as the keto-acid analogue of methionine,  $\alpha$ -keto- $\gamma$ -methiobutyric acid, is then produced. No methyl mercaptan was produced,



probably because under the experimental conditions it is rapidly autooxidized to the disulfide. From the cells three enzyme fractions have been isolated. One of these is the L-amino acid oxidase which produces the keto-acid analogue found when arsenite inhibition takes place. While  $\alpha$ -ketomethionine can be reconverted to methionine, it cannot be further degraded in these organisms. Secondly, there is a methionine racemase that converts either D- or L-methionine into a racemic mixture, pyridoxal phosphate serving as cofactor. The third enzyme is termed methionine dethiomethylase, and is apparently identical with a similar enzyme in *Clostridium sporogenes*. It also requires pyridoxal phosphate as a cofactor. Attacking only L-methionine, it irreversibly removes the thiomethyl group and ammonia, and leaves  $\alpha$ -ketobutyric acid. Arsenite completely inhibits this reaction, which is very similar to the action catalyzed by homocysteine desulfhydrase.

Among the anaerobic reactions of cysteine, we find that pyruvate and ammonia are produced in bacteria by the action of cysteine desulfhydrase. This enzyme removes  $\text{H}_2\text{S}$  and produces aminoacrylic acid, which is spontaneously hydrolyzed into the end-products. The possible reversal of this reaction in the organic incorporation of sulfate in animals has already been mentioned. Transamination of cysteine with  $\alpha$ -ketoglutarate by heart and liver preparations yields  $\beta$ -mercaptopyruvate ( $\text{SH}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH}$ ), a metabolically highly active compound. Animal tissues, as well as some bacteria, convert it to pyruvate and elemental sulfur. In *Endamoeba histolytica* a transamination between cysteine and pyruvic acid yields alanine, in addition to the  $\beta$ -mercaptopyruvate. Degradation of the latter to pyruvate and sulfur is coupled with carbohydrate metabolism, in such a way that hexosediphosphate and inorganic phosphate with elemental sulfur yield diphosphoglycerate and  $\text{H}_2\text{S}$ .

The conversion of cysteine to cystine is commonly catalyzed by cytochrome *c* and cytochrome oxidase, but there is also a spontaneous oxidation of cysteine by elemental sulfur:  $2 \text{RSH} + \text{S} \rightarrow \text{RSSR} + \text{H}_2\text{S}$ ; and there is a reduction of cystine to cysteine by DPNH in yeast and higher plants. The primary part of Singer's discussion dealt not so much with these as with the oxidation of cysteine via



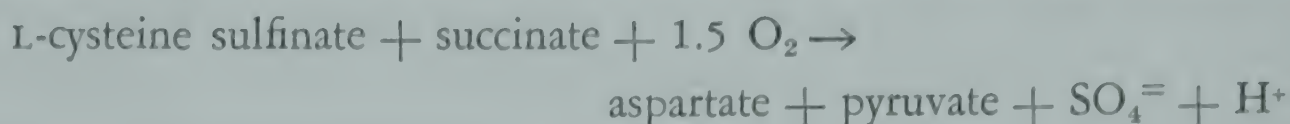
cysteine sulfinic acid ( $\text{SO}_2\text{H}\cdot\text{CH}_2\cdot\text{CHNH}_2\cdot\text{COOH}$ ), the principal pathway to the formation of inorganic sulfate from cysteine, cystine, and methionine. The postulated role of cysteine sulfinic acid in this pathway is supported by the observation that cysteine injected into rats is converted into hypotaurine ( $\text{SO}_2\text{H}\cdot\text{CH}_2\cdot\text{CH}_2\text{NH}_2$ ), which is known to be a decarboxylation product of cysteine sulfinic acid. Taurine ( $\text{SO}_3\text{H}\cdot\text{CH}_2\cdot\text{CH}_2\text{NH}_2$ ) is produced similarly from cysteic acid, perhaps by the very same decarboxylase. There is also tentative evidence of a conversion of hypotaurine into taurine.

The first extensive studies of cysteine sulfinic acid metabolism were carried out by Singer and Kearney in *Proteus vulgaris*. Here two alternative pathways exist. The more rapid reaction begins with a transamination with  $\alpha$ -ketoglutarate or oxaloacetate. The product,  $\beta$ -sulfinylpyruvate ( $\text{SO}_2\text{H}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH}$ ), is desulfinated in the presence of  $\text{Mn}^{++}$ , and the sulfite is rapidly and non-enzymatically oxidized to sulfate in the presence of  $\text{Mn}^{++}$ . The second pathway is that of the conversion of cysteine sulfinic acid to cysteic acid ( $\text{SO}_3\text{H}\cdot\text{CH}_2\cdot\text{CHNH}_2\cdot\text{COOH}$ ), followed by transamination of the cysteate with  $\alpha$ -ketoglutarate to yield  $\beta$ -sulfonylpyruvate ( $\text{SO}_3\text{H}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH}$ ). Both transaminations are exceedingly rapid, but the dehydrogenation of cysteine sulfinic acid to cysteate is relatively slow.

In rabbit liver preparations a desulfinase reaction occurs that suggests that the *Proteus vulgaris* scheme is but slightly modified. Cysteine sulfinic acid may be decarboxylated to hypotaurine, or may be desulfinated to yield alanine and  $\text{SO}_2$ . The latter reaction requires the participation of  $\alpha$ -ketoglutarate, which is regenerated in a double transamination system so that it appears as a cofactor. Singer's own studies were carried out with rat liver and beef heart mitochondrial preparations. With these, it was found that oxaloacetate could also serve as recipient in the transamination, and that the rapidity of the transamination to oxaloacetate rendered possible the efficient oxidation of the precursors of oxaloacetate (fumarate and malate) in a coupled reaction. Singer also reported in some detail on recent work with another reaction coupled with cysteine sulfinic acid, the oxidation of succinate. Mitochondrial preparations of a variety of mammalian and avian tissues will rapidly oxidize cysteine to



cysteate in the presence of DPN and an eluate factor which was identified as succinic acid. The overall reaction may be written:



Proofs of the participation of succinate in the reaction are that it is inhibited by malonate, and that the entire reaction sequence depends on the initial dehydrogenation of succinate. In the light of the studies previously described, it is clear that the succinate serves in this system to generate oxaloacetate, which is necessary for the transamination of cysteine sulfinic acid. Singer and Kearney have succeeded in purifying a soluble succinic dehydrogenase free of hemin compounds (and therefore of cytochrome *b*) that carries out initial step of the coupled reaction, the oxidation of succinate.

$\beta$ -Sulfinyl pyruvate, the primary product of these transaminations of cysteine sulfinic acid, does not accumulate in the mitochondrial extracts, but is converted to pyruvate and sulfite (beef heart preparations) or to pyruvate and sulfate (rat liver preparations).  $\beta$ -Sulfonylpyruvate ( $\text{SO}_3\text{H}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH}$ ) was excluded as a possible intermediate in the production of sulfate, and it was shown that the system, just as in *Proteus* and rabbit liver, undergoes cleavage of the substrate directly to  $\text{SO}_2$  and pyruvate. The final step is therefore that of the oxidation of sulfite to sulfate. Judging from results obtained in Handler's laboratory, this transformation is quite complex, requiring two enzymes as well as inorganic sulfate. In the discussion, Handler reported the isolation of one enzyme he suspects to be the real sulfite oxidase. It is a metallo-flavoprotein that will reduce methylene blue or cytochrome *c* in the presence of sulfite. In the absence of the cytochrome system, and while respiring aerobically, it generates  $\text{H}_2\text{O}_2$ . Singer and Kearney have found a sulfite oxidase in the rat liver mitochondria.

In addition to all the varieties of transaminative oxidation of cysteine sulfinic acid previously discussed, there is also a non-transaminative oxidation that requires DPN and an autooxidizable dye, and that yields pyruvate, sulfate, and ammonia. This system is likewise present in rat liver mitochondria, the enzyme being an anaerobic



pyridine nucleotide dehydrogenase analogous to glutamic dehydrogenase.

All these studies of cysteine sulfinic acid indicate it to be a highly active metabolite in animals as in microorganisms, to yield the same end-products aerobically as cysteine, namely, pyruvate and inorganic sulfate, and to be on the main pathway of the oxidation of cysteine. The demonstration that inorganic sulfate in animal tissues arises from cysteine sulfinic acid confirms Pirie's hypothesis of twenty years ago.

### *The Synthesis of Threonine ( $\text{CH}_3 \cdot \text{CHOH} \cdot \text{CHNH}_2 \cdot \text{COOH}$ )*

Recent knowledge of the synthesis of threonine from aspartic acid indicates the existence of four steps, as shown in Fig. 10, taken from the review contribution to the symposium by Black and Wright. Isotope studies with microorganisms, the analysis of the blocks in threonine-requiring mutants, and the isolation of enzyme systems from yeast and from several *E. coli* mutants have all contributed to this picture. One mutant of *E. coli*, with a block between homoserine and threonine, accumulated the former compound. In *Neurospora* there is a mutant that can utilize homoserine in place of threonine in the medium, and a similar mutant has been found in *E. coli*. Threonine was shown to exert a sparing action on the requirement for aspartate in lactobacilli. Isotopic tracers in acetate turned up in identical distributions in threonine and aspartic acid; and aspartate labeled in carbon-3 yielded threonine labeled mainly in carbon-3. These results, obtained by a number of investigators, were suggestive but not conclusive. The evidence from the isolated enzyme systems, reported by Black, has completed the necessary evidence.

The yeast enzyme systems have yielded the two intermediates,  $\beta$ -aspartyl phosphate and aspartic  $\beta$ -semialdehyde. These have been isolated and compared with the synthetic compounds. The  $\beta$ -aspartyl phosphate is very labile. It is produced from L-aspartate by an enzyme called  $\beta$ -aspartokinase, which requires ATP and  $\text{Mg}^{++}$  ions for activity. The second intermediate is produced from  $\beta$ -aspartyl phosphate by the enzyme L-aspartic  $\beta$ -semialdehyde dehydrogenase, which requires TPNH as a cofactor. The third enzyme in



the system is L-homoserine dehydrogenase, which converts the semi-aldehyde to L-homoserine. DPNH is the cofactor for this step. The *E. coli* mutants studied at the Pasteur Institute have revealed exactly the same steps. The French workers have advanced the knowledge of step 4 in particular, by showing that the conversion of homoserine to threonine is dependent upon both ATP and pyridoxal phosphate.

#### INTERMEDIATE STEPS IN THE BIOSYNTHESIS OF THREONINE

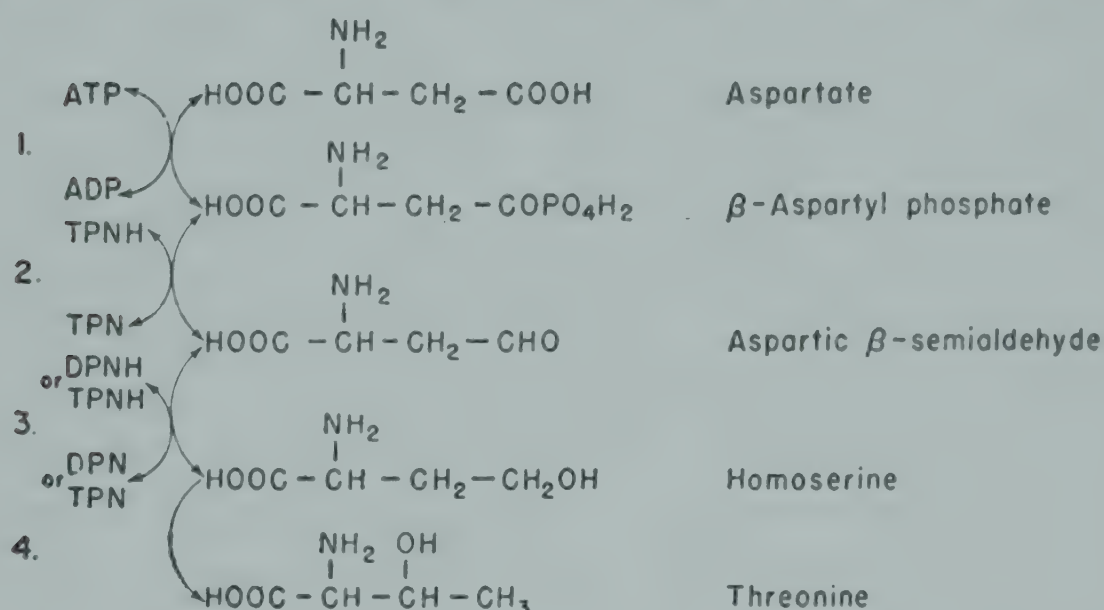


FIG. 10. Steps in the biosynthesis of threonine in yeast and in *Escherichia coli*. (From Black and Wright.)

There is probably at least one intermediate between homoserine and threonine, for hydroxylamine reacts with homoserine in the presence of ATP to form a hydroxamic acid, the usual criterion of the presence of an activated intermediate; and also the disappearance of homoserine fails to parallel the formation of threonine.

#### THE METABOLISM OF GLYCINE AND SERINE

The versatility of glycine and serine, and their frequent appearance as precursors and end-products in numerous pathways station them truly at a major crossroad of metabolism.

#### *Glycine* ( $\text{CH}_2\text{NH}_2\cdot\text{COOH}$ ) and *Glyoxylic Acid* ( $\text{CHO}\cdot\text{COOH}$ )

Weinhouse particularly concerned himself in the initial paper of this session with the interconvertibility of glycine and glyoxylic acid. In photosynthesis,  $\text{C}^{14}$  in labeled  $\text{CO}_2$  goes quickly into glycolic acid

( $\text{CH}_2\text{OH}\cdot\text{COOH}$ ) and glycine. In the rat, the rapidity with which glyoxylate and glycolate are converted into glycine is equally noteworthy. Oxalic acid ( $\text{COOH}\cdot\text{COOH}$ ), which is not metabolized, is formed readily from glyoxylic acid, but Weinhouse's experiments with rat liver preparations show that this occurs only at high concentrations of glyoxylate. At physiological concentrations, glyoxylic acid does not yield oxalate, but is entirely oxidized via formate to  $\text{CO}_2$ . This fact removes one of the chief objections to regarding glyoxylic acid as a product of glycine metabolism, namely, that glycine normally does not give rise to oxalic acid. The formate is produced exclusively from the  $\alpha$ -carbon atoms of glycine, glyoxylic acid, and glycolic acid, and not at all from the carboxyl carbons.

The primary reaction in the formation of glycine was thus established to be a transamination between glyoxylate and a considerable number of amino acids and amines. This transamination occurred quite rapidly even without an enzyme, but enzymatically was about ten times as fast. The transaminase system is apparently non-reversible, since glycine and  $\alpha$ -ketoglutarate undergo relatively little exchange. In liver and kidney glycine is oxidatively deaminated to glyoxylic acid. Step 2, the conversion of glyoxylic acid to formic acid and  $\text{CO}_2$ , ( $\text{CHO}\cdot\text{COOH} + \frac{1}{2}\text{O}_2 \rightarrow \text{HCOOH} + \text{CO}_2$ ), is not well characterized as yet, although it too goes on non-enzymatically at a very rapid rate. The side-reaction from glyoxalate to oxalic acid ( $\text{CHO}\cdot\text{COOH} + \frac{1}{2}\text{O}_2 \rightarrow \text{COOH}\cdot\text{COOH}$ ) is catalyzed by xanthine dehydrogenase, and presumably by some different enzyme in pigeon liver homogenates, where it occurs in the absence of that enzyme. Step 3 in the system, the oxidation of formic acid, is probably carried out by a catalase-hydrogen-peroxide complex ( $\text{HCOOH} + \text{H}_2\text{O}_2 \rightarrow \text{CO}_2 + 2\text{H}_2\text{O}$ ).

Further experiments were conducted by Weinhouse and his co-workers to determine the relative importance of the glyoxylate pathway of glycine oxidation in comparison with the pathway through serine and pyruvate. It appears that both the glycine  $\alpha$ -carbon and the serine  $\beta$ -carbon contribute very little to acetoacetate via pyruvate, whereas formate is a major product of both glycine and serine in rat liver. The glycine-serine interconversion was rapid, however; so



that probably the major pathway for the oxidation of serine is through glycine to glyoxalate and formate. Ribose-5-phosphate is reported by Weissbach and Horecker to give rise to glycine through the means of a transketolase that splits the pentose so as to yield glycolaldehyde ( $\text{CH}_2\text{OH}\cdot\text{CHO}$ ); and glycolaldehyde can also arise from fructose-6-phosphate through the action of transketolase (Racker et al.). Thus glyoxylate and glycine may readily be derived from common foodstuffs. The synthesis of glycine from citric acid, as in *Pseudomonas aeruginosa*, may also be fairly widespread.

The recent studies have greatly amplified our knowledge of the metabolic pathways involving glycine, but, as in many other aspects of amino acid metabolism, the relative importance of these paths is not yet clearly demonstrable. Weinhouse fittingly concluded his discussion with the remark: "An important future goal in biochemistry is an understanding of the factors which direct and regulate the flow of metabolites through available metabolic channels."

#### *The Interrelationship between Glycine and Serine*

The conversion of serine to glycine was first recognized by Knoop exactly 40 years ago, and the interconversion of the two amino acids became apparent 10 years ago when mutants were found in *E. coli* with a nutritional requirement that could be satisfied by either serine or glycine. Tracer studies carried out in rats, guinea pigs, and yeast (*Torulopsis*) completed the evidence for an extensive and rapid interconvertibility of glycine and serine, and raised the question of the origin of the one-carbon unit required to convert glycine into serine.  $\text{CO}_2$  was shown to be unutilizable for this purpose in rats, and although both formate and formaldehyde are incorporated into serine, the evidence indicated that neither of these was directly involved, but rather that there was some intermediate common to both.

Pyridoxal phosphate is a requisite for serine biosynthesis in various microorganisms and in chicks, and it has been proposed that it forms a Schiff base with glycine and serine so as to facilitate the interconversion. There is now a great deal of evidence to implicate folic acid and its derivatives, such as tetrahydrofolic acid, in transfers of



one-carbon units, including that to serine. For example, folic-acid-deficient rats incorporate formate into the  $\beta$ -carbon atom of serine very poorly, and the conversion of serine to glycine is likewise impaired. Because removal of the  $\beta$ -carbon of serine does not involve an obligatory oxidation to a formyl group, the *citrovorum* factor itself—formyltetrahydrofolic acid—seemed unlikely to be the active carrier of the one-carbon unit; and several workers (Welch and Nichol; Sprinson—see above) have proposed that there is also a hydroxymethyl form of tetrahydrofolic acid. Evidence in favor of this hypothesis was obtained by Sakami and his coworkers. They found that in pigeon liver and rat liver preparations the incorporation of a one-carbon unit derived from the  $\beta$ -carbon of serine into carboxyl-labeled glycine so as to produce carboxyl-labeled serine was markedly increased by adding tetrahydrofolic acid, but not by adding ATP, pyridoxal phosphate, or homocysteine. One may therefore envisage a condensation between the hydroxymethyltetrahydrofolic acid and activated glycine, followed by a hydrolytic cleavage yielding serine. The utilization of formaldehyde in synthesizing serine in pigeon liver extracts and washed rat liver particles was similarly influenced by tetrahydrofolic acid. On the other hand, the incorporation of formate into the  $\beta$ -carbon of serine, inasmuch as that would involve a reduction of the one-carbon unit from the oxidation level of formate to that of formaldehyde, is not stimulated by tetrahydrofolic acid alone, but only if the latter is accompanied by ATP, DPN, glucose-6-phosphate, and  $Mn^{++}$ . Thus the data fit the hypothesis that the formate is incorporated into the formyltetrahydrofolic acid (*citrovorum* factor) with the aid of ATP, and that it is then reduced to the hydroxymethyltetrahydrofolic acid with the assistance of DPNH. The transfer of the  $\beta$ -carbon of serine to the methyl group of choline or thymine may readily proceed via the hydroxymethyltetrahydrofolic acid, without oxidation to a formyl intermediate; and this again agrees with the existing evidence. It seems probable that the active  $C_1$ -carrier is a bound form of the folic acid derivative, perhaps a polyglutamate, and that the well-known inhibition by aminopterin of the metabolism of one-carbon units is at the level of the conversion of folic acid to the tetrahydro form.



Sakami concluded his review of the interconversions of glycine and serine by pointing out that while these are rapid and take place in virtually all animal tissues that have been examined, there is still no knowledge of the net balance of the process. Perhaps it varies from time to time, with need, and according to tissue. At any rate, this admission reinforces the concluding remark made by Weinhouse, quoted above.

In dog and rabbit liver, and to a lesser extent in dog kidney there has been found, according to Sallach, a transaminase that forms serine from hydroxypyruvic acid and alanine. Acetone-powder extracts have far more activity than homogenates. As required if the interpretation of a transamination reaction is correct, equimolar quantities of pyruvate and serine are formed in the reaction, and the total amount of  $\alpha$ -keto acids reducible by muscle lactic dehydrogenase remained constant. Pyridoxal phosphate, as in the case of known transaminations, functions as cofactor in this system. Alanine was the only donor amino acid. The high activity of the hydroxypyruvic-alanine transaminase in liver and kidney marks this as a major pathway of serine metabolism in these tissues.

An extension of the same topic was supplied by Mackenzie's description of studies of the formation of sarcosine (monomethylglycine,  $\text{CH}_3\text{—NH}\cdot\text{CH}_2\cdot\text{COOH}$ ) from dimethylglycine and betaine, and the conversion of sarcosine into serine through the incorporation of an active one-carbon unit. In what Mackenzie calls a methyl oxidation cycle, starting with betaine [ $(\text{CH}_3)_3\text{—}\overset{+}{\text{N}}\cdot\text{CH}_2\cdot\text{COOH}$ ], one methyl group is lost by transmethylation. Dimethylglycine [ $(\text{CH}_3)_2\text{—N}\cdot\text{CH}_2\cdot\text{COOH}$ ] loses another methyl group, which becomes an active one-carbon unit that may be oxidized to formaldehyde and formate, or may be transferred to glycine to synthesize serine. Sarcosine is next deprived of the remaining methyl group, which similarly may be oxidized to formaldehyde and formate or may go as an active one-carbon unit into serine. By decarboxylation, serine gives rise to ethanolamine ( $\text{NH}_2\text{—CH}_2\cdot\text{CH}_2\text{OH}$ ); and this compound, by repeated methylations, becomes converted into choline, which completes the cycle by being converted in turn into betaine

(Fig. 11). Thus on the left side of the cycle the synthesis of the active methyl donors proceeds, and on the right there occurs the generation of active one-carbon compounds.

The existence of this cycle is based on an extensive body of evidence derived from experiments with animal tissues, in particular with rat liver and kidney preparations and with living animals. Whereas both the dimethylglycine oxidase and sarcosine oxidase are present in the liver of the rat, hamster, rabbit, and guinea pig,

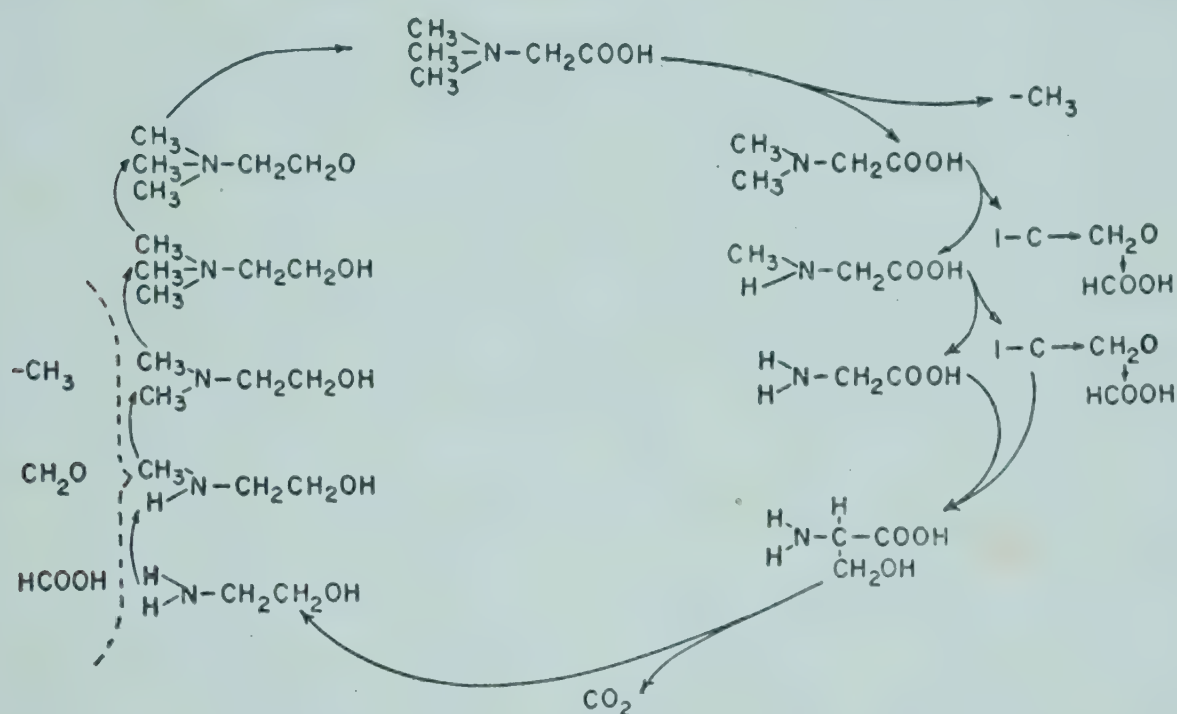


FIG. 11. The methyl transfer cycle and the generation of one-carbon units. (From Mackenzie.)

there is very little of either in chicken liver. Further comparative studies will be needed to show whether it is safe to generalize that dimethylglycine and sarcosine represent a major pathway for the oxidation of N-methyl groups in the mammal but not in birds.

According to the results of isotope studies, the  $\beta$ -carbon of serine is derived in these experiments exclusively from the methyl carbon of sarcosine, and the other two carbons of serine come from their counterparts in the glycine moiety of sarcosine. In other words, although others have reported on the extensive incorporation of the  $\alpha$ -carbon of glycine into serine, in this mitochondrial system the  $\beta$ -carbon of serine is not derived from that source. Exogenous glycine can be made to substitute in the reaction for the glycine derived



from sarcosine. The active one-carbon unit ("active formaldehyde") is not formaldehyde itself, nor is it the aminolevulinic acid of the succinate-glycine cycle (see Shemin, below). It possesses the oxidation level of formaldehyde and may be converted back to a methyl group without ever attaining the oxidation level of formate. This appears to fit very well with that description of the hydroxymethyl form of tetrahydrofolic acid as the carrier of the active one-carbon unit which has been made in previous discussions (Sakami; Sprinson). Added DPN leads to the oxidation of free formaldehyde but not of the "active formaldehyde"; flavin-adenine-dinucleotide catalyzes the oxidation of both. The serine-synthesizing enzyme was shown to be distinct from that which condenses pyruvate and formaldehyde to  $\alpha$ -keto- $\gamma$ -hydroxybutyric acid. The fact that the addition of cysteine to the system inhibits the formation of formaldehyde but does not alter the synthesis of serine affords yet another indication of the distinction between formaldehyde and "active formaldehyde." Both cysteine and homocysteine react with free formaldehyde but not with the "active formaldehyde."

Mackenzie emphasizes that the cycle he has depicted should only be regarded as the "backbone of a much more complicated system of metabolic interrelationships," such as those discussed by Weinhouse and Sakami in the present symposium. A direct oxidation of dimethylaminoethanol to dimethylglycine, would, for example, constitute a shunt across the cycle. The methyl group transferred to methionine from betaine may reenter the cycle in the methylation of the aminoethanols; and this cycle certainly does not represent the sole pathway for the metabolism of the methyl group of methionine. Nonetheless, this "represents a major and important pathway, not only for the conversion of methyl groups to carbon dioxide, but also for the generation of considerable quantities of one-carbon compounds, which, by their extensive participation in synthetic reactions, earns for many of the methyl carbons a brief respite from their ultimate metabolic fate, the formation of carbon dioxide and water."

### *The Succinate-Glycine Cycle*

Yet another new cycle to be described was that presented by

Shemin. This cycle couples glycine metabolism and the formation of active one-carbon units with the tricarboxylic acid (citric acid; Krebs) cycle. It is well represented in the scheme given by Shemin (Fig. 12).

The cycle commences with a postulated condensation of "activated" succinic acid and glycine to yield  $\alpha$ -amino- $\beta$ -ketoadipic acid, which is then decarboxylated to  $\delta$ -aminolevulinic acid. This impor-

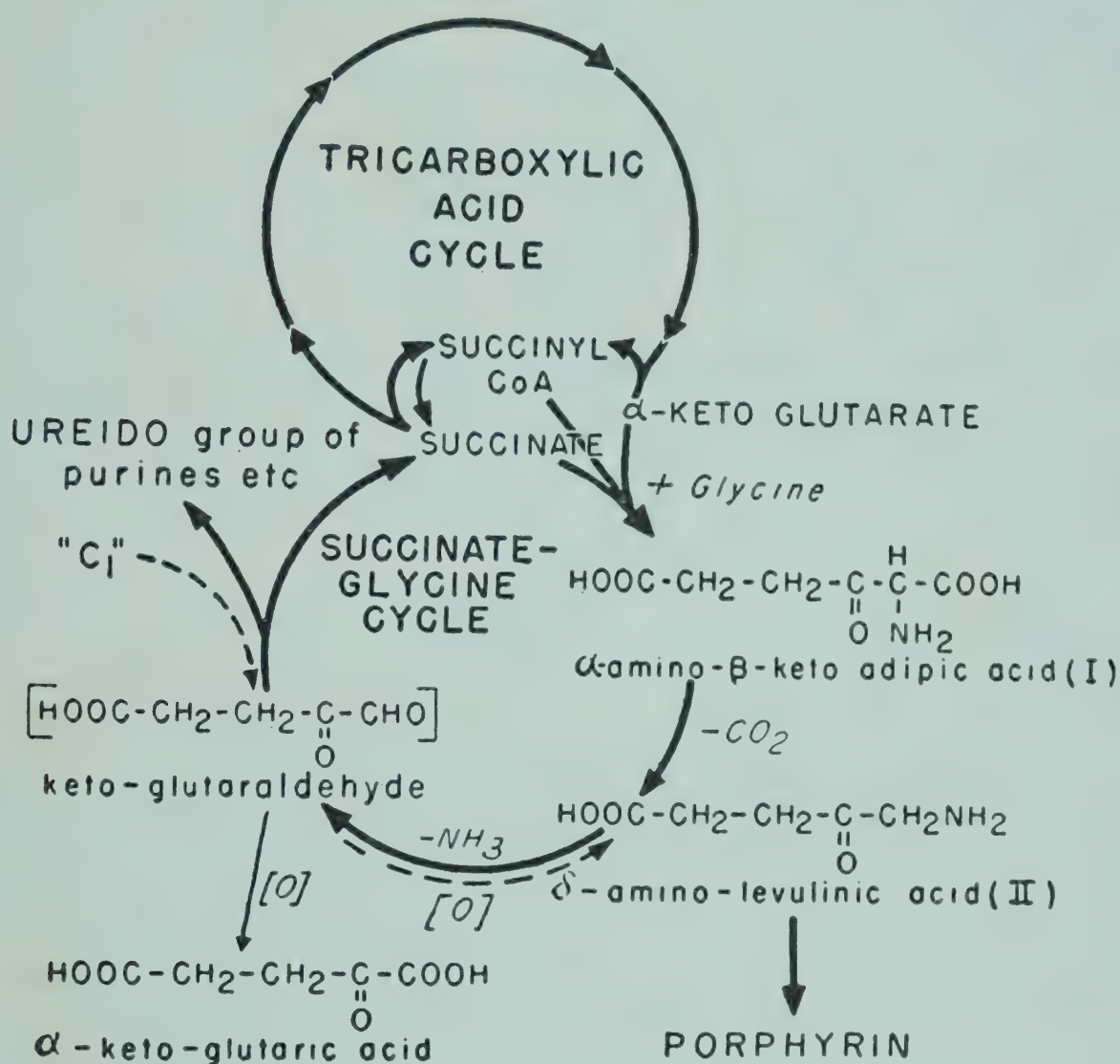


FIG. 12. The succinate-glycine cycle: a pathway for glycine metabolism. (From Shemin.)

tant new intermediate compound may be used in porphyrin synthesis or be deaminated to ketoglutaraldehyde, which may re-enter the citric acid cycle either by oxidation to  $\alpha$ -ketoglutarate or, after removal of an active one-carbon unit, by conversion to succinic acid. The  $\text{C}_1$  unit can be incorporated into 5-amino-4-imidazolecarboxamide to complete the ureido structure of the purine bases.

This postulated scheme grew out of the effort to find out how the porphyrin ring structure is synthesized. It had earlier been demon-



strated that the porphyrin ring is derived from glycine and succinic acid, 8 of the carbon atoms and all 4 of the nitrogen atoms being derived from glycine. The glycine carboxyl group is not used at all. The remaining 26 carbon atoms come from succinate. From the labeling patterns, each pyrrole unit was found to be made up of two moles of succinate and one of glycine, and the methene bridges connecting the pyrrole rings are also derived exclusively from glycine (see Fig. 13).

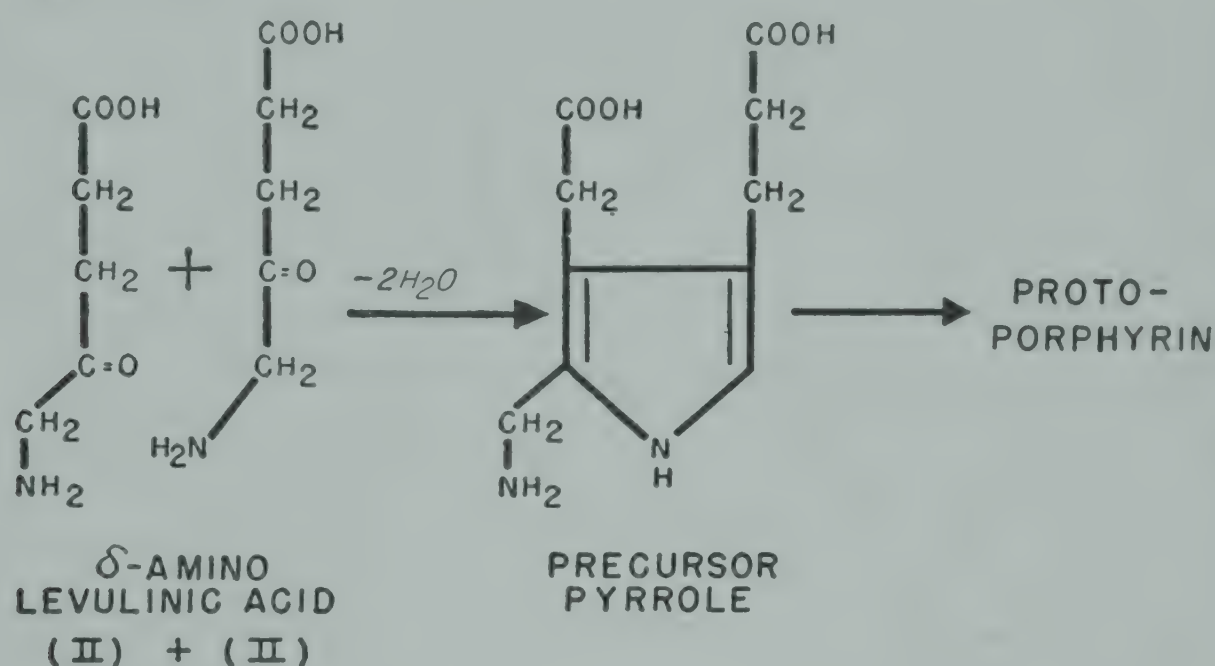


FIG. 13. The formation of the precursor pyrrole for porphyrins from two moles of  $\delta$ -aminolevulinic acid. Since the four upper carbons of each  $\delta$ -aminolevulinic molecule come from succinate and the lower carbon and the amino group of each come from glycine, the diagram also illustrates the location of the glycine moieties in the pyrrole rings and the methene bridges of the porphyrin structure. (From Shemin.)

Experiments were carried out with whole or hemolyzed red blood cells of a duck in such a way that the individual carbon atoms from particular positions in the porphyrin ring could be identified. The first striking conclusion was that the  $\alpha$ -carbon atom of glycine is used *equally* for the pyrrole ring carbon and the methene bridge carbon. It would therefore appear that the same derivative of glycine is used for both positions. A consideration of the limited possibilities then led to the concept of a condensation of succinate on the  $\alpha$ -carbon of glycine, so as to yield  $\alpha$ -amino- $\beta$ -ketoadipic acid; and the latter compound, being a  $\beta$ -keto acid, could readily be decarboxylated to form  $\delta$ -aminolevulinic acid. By a Knorr type of condensation, two moles of this intermediate compound could condense to form a

pyrrole ring (Fig. 13). The experiments demonstrated that  $\delta$ -aminolevulinic acid is utilized for porphyrin formation; and, as expected on the basis of the hypothesis, when  $\delta$ -aminolevulinic acid was labeled in carbon-5, the positions of the labeled carbon atoms in the porphyrin were the same as in porphyrin synthesized from labeled glycine. Moreover, the precursor pyrrole that would be formed by the condensation of two atoms of  $\delta$ -aminolevulinic acid has the same structure as porphobilinogen, which is excreted in the urine of patients with acute porphyria. Porphobilinogen thus becomes established as an intermediate in the synthesis of porphyrin.

The condensation of succinate and glycine occurs only within the intact red cell or in cells hemolyzed with water, and not in homogenized preparations.

Since the  $\delta$ -carbon atom of the aminolevulinic acid is derived from the  $\alpha$ -carbon of glycine, it might be predicted that it would also become the active one-carbon unit required for the synthesis of the purine ring or the methyl group of methionine. By labeling this carbon atom in  $\delta$ -aminolevulinic acid, it was demonstrated that it does participate in these reactions. Another point of interest is that the cycle provides ways in which the carbon atoms of glycine may be oxidized to  $\text{CO}_2$ . The carboxyl carbon atom of glycine leaves the cycle in the decarboxylation of  $\alpha$ -amino- $\beta$ -ketoadipic acid; the  $\alpha$ -carbon atom may end up as the carboxyl carbon of  $\alpha$ -ketoglutarate and is removed in the decarboxylation of  $\alpha$ -ketoglutarate to succinic acid. In this way both carbon atoms of glycine can be completely oxidized to  $\text{CO}_2$  without passing through formic acid.

Shemin has further suggested that the condensation of succinate with glycine may be only one example of a general type of condensation reaction. For example, "active" acetate can compete with succinate for glycine in this system, the analogue aminoacetone then being formed instead of  $\delta$ -aminolevulinic acid. It has been shown that aminoacetone can also provide active one-carbon units for the synthesis of purines, but there is no proof as yet that aminoacetone is synthesized by the cell. Shemin also suggests that the route via  $\alpha$ -amino- $\beta$ -ketoadipic acid is the normal pathway whenever the entire glycine molecule is utilized. Serine too might be synthesized from



glycine in this way, if  $\alpha$ -amino- $\beta$ -ketoadipic acid were to react with an active one-carbon unit and yield serine and succinic acid.

### *Contributions of the Amino Acids to Purine Synthesis*

Isotope tracer studies have shown that the nitrogen atoms 1, 3, and 9 of the purine ring (Fig. 14) come from aspartic acid, glutamic acid, or glutamine. Glycine, which supplies carbon atoms 4 and 5, supplies also nitrogen atom 7. Carbon atom 6 is derived from  $\text{CO}_2$ , and the remaining carbon atoms (2 and 8) are supplied by formate

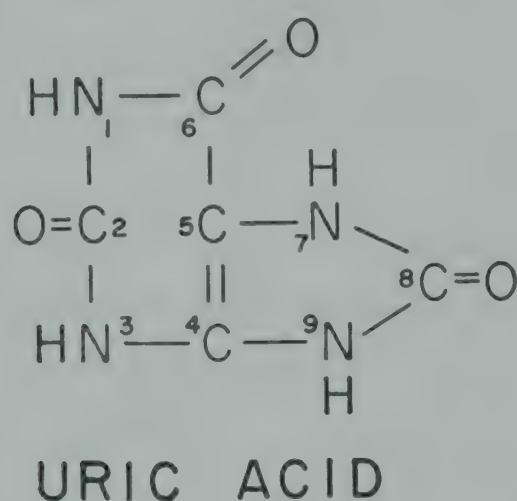


FIG. 14. The uric acid molecule, labeled to show the numbering of the atoms in the purine ring.

or active one-carbon units. Buchanan and his coworkers report that in furnishing nitrogen atoms 1 and 3 there must apparently be a coupled relation between an amino acid and an amide, for the combinations glutamine plus aspartic acid, and asparagine plus glutamic acid, were far more effective than either single amino acids or amides, or combinations of an amino acid and its own amide analogue. Nitrogen atom 9 is derived from an amide nitrogen, viz., from glutamine. The inhibitor of purine biosynthesis, azaserine, causes a drastic reduction in the amount of product when the combination asparagine and glutamic acid is used in the synthesis.

A number of glycine derivatives have been tested in regard to utilization in the synthesis of the purine ring, and all have been excluded. It consequently seems that the ribotidation of glycine must occur at a very early stage in purine synthesis. This is also indicated by the evidence (G. R. Greenberg) that when radioactive formate

is used in purine synthesis, inosinic acid has a higher specific activity than either inosine or hypoxanthine, a fact indicating that it is a precursor of those purines and that a ribosyl-5-phosphate moiety is added to some purine precursor before the purine ring is completed. Buchanan and his coworkers have now studied the exchange reaction leading to the incorporation of formate into position 2 of the ring. The purine ring of inosinic acid (but not of inosine or hypoxanthine) can undergo cleavage to 5-amino-4-imidazolecarboxamide ribotide, the one-carbon unit released thereupon undergoes equilibration with radioactive formate, and the purine ring is then reformed with radioactive carbon in position 2. Buchanan proposes that the mechanism is that of a two-step reaction. In step 1, the purine ring cleaves between atoms 1 and 2, leaving a formyl group at position 2. In step 2, catalyzed by a transformylase, this group is transferred to a cofactor, and 5-amino-4-imidazolecarboxamide ribotide remains. The cofactor is not clearly identified, since these workers have obtained two enzyme fractions, one of which carries out the transformylation without any additions, whereas the other is stimulated by the addition of formyl-tetrahydrofolic acid (leucovorin) and  $\text{Cu}^{++}$ . This fact leaves the participation of the folic acid derivative in the process uncertain; but Goldthwait and his coworkers reported that the citrovorum factor was converted to the active cofactor by ATP. Glycine does act in this system as a formyl (or hydroxymethyl) acceptor, as one would expect, and the addition of glycine stimulates the reaction. When the reaction goes in the opposite direction, presumably serine can serve as the source of the active formyl group, which is transferred from the coenzyme to the carboxamide so as to complete the purine ring.

Goldthwait, Peabody, and Greenberg, have proposed some additional steps in the synthesis of the purine ring. Two glycine ribotides have been isolated chromatographically in a pigeon liver system, and also an aminoimidazole ribotide from a purine-requiring mutant strain of *E. coli* (Chamberlain et al.). Compound I (Fig. 15) seems to be a simple glycine amide ribotide, which must precede the formation of Compound II, which is the same compound formylated. In their study of the transfer of the formyl group to the carboxamide



ribotide, Goldthwait and his coworkers found not only that formyl-tetrahydrofolic acid functions as the active cofactor, but also that dihydrofolic acid when accompanied by DPNH could also fix the

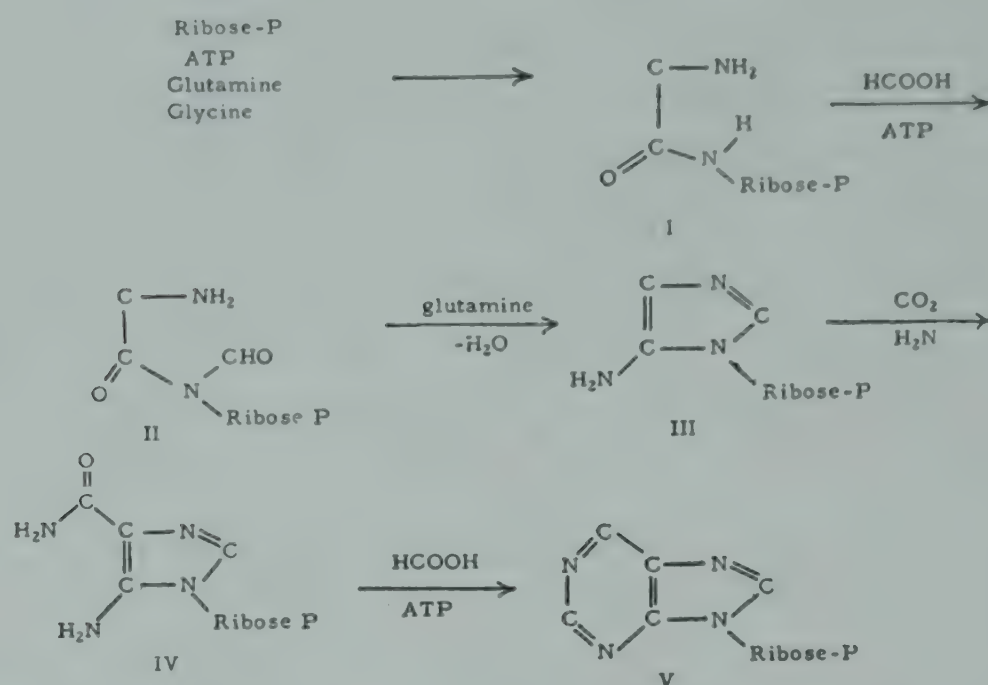


FIG. 15. Proposed scheme for purine biosynthesis, based on data from yeast and *Escherichia coli*. The position of the formyl group in Compound II is unknown. (From Goldthwait, Peabody, and Greenberg.)

formyl group in the purine ring. Whereas ATP is necessary in the formation of the active cofactor, it is not required for the transfer of the formyl from the latter to the carboxamide compound. The

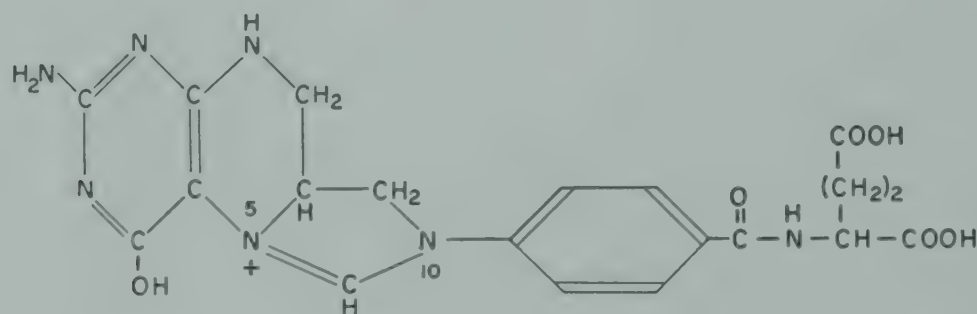


FIG. 16. The proposed bridge structure of anhydroleuovorin, the intermediate in the transfer of the formyl group from N<sup>5</sup> in the activated citrovorum factor (N<sup>5</sup>-formyl-tetrahydrofolic acid) to N<sup>10</sup>-formyl-tetrahydrofolic acid. (After Goldthwait et al.)

active cofactor has the formyl group shifted from nitrogen atom 5, its attachment in the citrovorum factor, to nitrogen atom 10. An intermediate bridge form (Fig. 16), called anhydroleuovorin, has been prepared chemically and tested for activity. It reacted very specifically with the carboxamide ribotide, and not with the

riboside or free base. Either this form or the N<sup>10</sup>-formyltetrahydrofolic acid appears likely to be the active cofactor. ATP is presumably involved in the formation of the bridge structure, at which time one of the nitrogens must carry a positive charge.

The stage at which CO<sub>2</sub> is incorporated into the purine ring structure at position 6 remains uncertain, but it may well be a condensation on Compound III, the amino imidazole ribotide. Goldthwait et al., assuming it to be unlikely that CO<sub>2</sub> per se is fixed into the precursor, have performed some experiments to determine whether or not succinate might be used, according to the proposal of Shemin. However, succinate was found to be far less efficient a precursor than CO<sub>2</sub> itself.

## THE METABOLISM OF THE AROMATIC AMINO ACIDS

### *Biosynthesis of the Aromatic Amino Acids*

Mutants of *E. coli* and *Neurospora* are known which have a quadruple nutritive requirement for tyrosine, phenylalanine, tryptophan, and para-aminobenzoic acid. Davis, who has carried out extensive studies of these auxotrophs in *E. coli*, has found that most of them also exhibit a requirement for para-hydroxybenzoic acid and for a sixth, still unidentified, growth factor. The existence of these multiple requirements implies the existence of common precursors in the synthetic paths leading to the aromatic amino acids. The identification of substances accumulated by auxotrophs which are blocked at different stages in the synthesis, or of growth factors demonstrable by crossfeeding between different mutant strains blocked at different points, has led Davis and his coworkers to define a number of steps centering on the synthesis of a common precursor, shikimic acid, a compound formerly known only as a rare acid in certain plants. These pathways are illustrated in Fig. 17.

The immediate precursor of shikimic acid, 5-dehydroshikimic acid, is accumulated in certain mutants and serves as a growth factor for mutants with still earlier blocks. The enzyme that reduces this to shikimic acid specifically requires TPN as coenzyme. The reaction is reversible. A mutant blocked between dehydroshikimic acid and shikimic acid lacks the enzyme.



The enzyme 5-dehydroquinase, which converts 5-dehydroquinic acid to 5-dehydroshikimic acid, also catalyzes a reversible reaction. No cofactor appears to be necessary. Again, no enzyme could be detected in mutants blocked at this step.

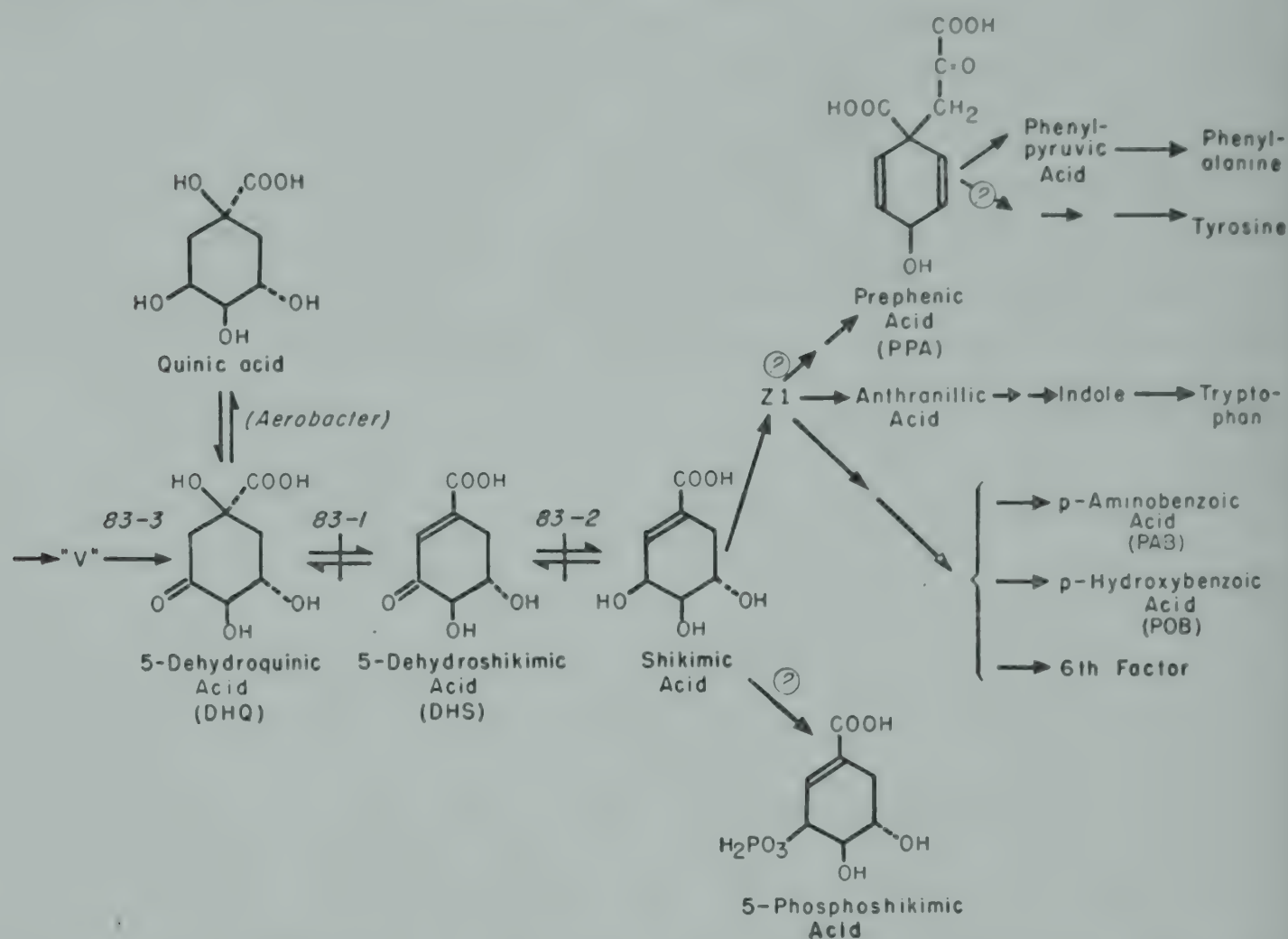


FIG. 17. The biosynthesis of the aromatic amino acids and other related compounds from shikimic acid and its precursors. (From Davis.)

Quinic acid is placed to the side of the main pathway because the *E. coli* mutants that responded to dehydroquinic acid neither responded to quinic acid nor accumulated it. Only in *Aerobacter aerogenes* is there known to be an enzyme, quinic dehydrogenase, that carries out this conversion, and no mutant that blocks the reaction has been found. This enzyme has a specific requirement for DPN as cofactor.

Further studies have been initiated to detect still earlier stages in the synthetic pathway preceding dehydroquinic acid. By an ingenious method, which involves the incubation of filtrates of a mutant blocked somewhere before dehydroquinic acid with an extract of the strain blocked between dehydroshikimic and shikimic acids, it was

found that considerable quantities of dehydroshikimic acid could be produced from some precursor that had accumulated in the filtrate of the mutant with the earlier, unknown block. Sprinson has carried this analysis further by means of variously labeled D-glucose and D-xylose. Labeled carbon atoms from glucose went into all seven of the carbon atoms of shikimic acid without equilibration, a fact that rules out the general pathway of glycolysis as a route to shikimic acid. Again, since carbon-1 of glucose went extensively into carbon-2 of shikimic acid, whereas in the oxidative pathway of glucose metabolism via pentose and sedoheptulose the glucose-1-C is lost, the oxidative path also cannot be the sole route. Nevertheless, D-xylose does supply carbon to the shikimic acid molecule. Sprinson suggests that sedoheptulose might nevertheless contribute to the shikimic pathway because 7-carbon sugars might be formed by mechanisms that do not wholly depend on the pentoses but could incorporate the glucose-1-C from some other source. This hypothesis was confirmed in another paper in the symposium. Kalan and Srinivasan showed that incubation of a cell-free extract of the mutant strain blocked between dehydroshikimic and shikimic acids with simple glucose failed to yield a significant formation of dehydroshikimic acid; but incubation with phosphorylated sugars was successful. Since the utilization of glucose for the synthesis of shikimic acid implies that some 7-carbon compound must be an intermediate, sedoheptulose-7-phosphate was tried. The result was unsuccessful; but an observation that hexose diphosphate increased the yield from the heptose led to a trial of sedoheptulose-1,7-diphosphate, and this proved to be an excellent substrate. DPN serves as cofactor.

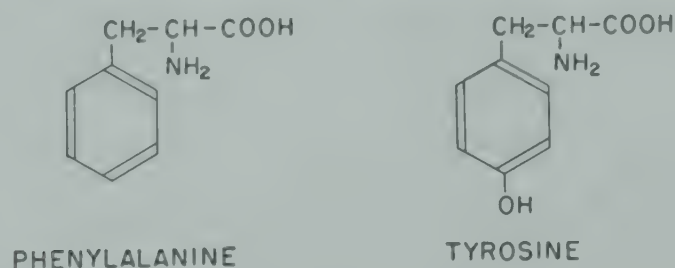
In the other direction from shikimic acid, Davis presented evidence for the occurrence of three compounds which are completely devoid of nutritional activity: 5-phosphoshikimic acid, an unidentified compound "Z1," and prephenic acid. The first of these is held to lie off the main synthetic pathway because not one among 59 mutants blocked somewhere along the chain of common precursors has been found to be blocked between shikimic and phosphoshikimic acids. Z1, on the other hand, is provisionally placed on the main pathway, just after shikimic acid, because there are some mutants that accumu-



late a lot of it, together with traces of shikimic and phosphoshikimic acids, while others accumulate shikimic and phosphoshikimic acids but no Z1.

Prephenic acid is accumulated by mutants that require phenylalanine or phenylpyruvic acid for growth. It is very labile, especially in an acid medium, where it is converted to phenylpyruvic acid. It is also converted into phenylpyruvic acid enzymatically, and this enzyme is lacking in some of the phenylalanine auxotrophs. This is an important point in supporting the position of prephenic acid as a true intermediate in phenylalanine synthesis, in spite of its complete inability to support growth in phenylalanine-requiring strains when it is supplied in the medium. Gilvarg has reported on the structure of prephenic acid. It has both the pyruvic side-chain and a carboxyl group attached to carbon-1 of the ring. The quaternary structure of this carbon atom excludes an aromatic structure for the six-carbon ring, and this must be converted to the aromatic ring of phenylpyruvic acid and phenylalanine. There is evidence of a hydroxyl group in para position on the ring. The removal of this hydroxyl group would aid in decarboxylation and in the assumption of the aromatic ring structure.

### *The Degradation of Phenylalanine and Tyrosine*



Knox's thorough review of the metabolism of these two aromatic amino acids shows that recent work has confirmed in all essentials the scheme proposed by Neubauer in 1909. Most insight into the oxidative degradation of tyrosine, which is formed from phenylalanine by hydroxylation, has been gained from the analysis of alcaptonuria, a human hereditary condition in which homogentisic acid (2,5-dihydroxyphenylacetic acid) is excreted in the urine instead of being broken down to acetoacetate. Neubauer postulated that tyrosine is converted to para-hydroxyphenylpyruvic acid, the keto acid

analogue of tyrosine, that this is then changed to 2,5-dihydroxyphenylpyruvic acid, and that this last compound is oxidatively decarboxylated to homogentisic acid. He also postulated that tyrosine might be converted to 2,5-dihydroxyphenylalanine and thereafter be deaminated, thus reaching 2,5-dihydroxyphenylpyruvic acid by an alternative path; but recent work has shown that although 2,5-dihydroxyphenylalanine can be metabolized in the animal, it is not

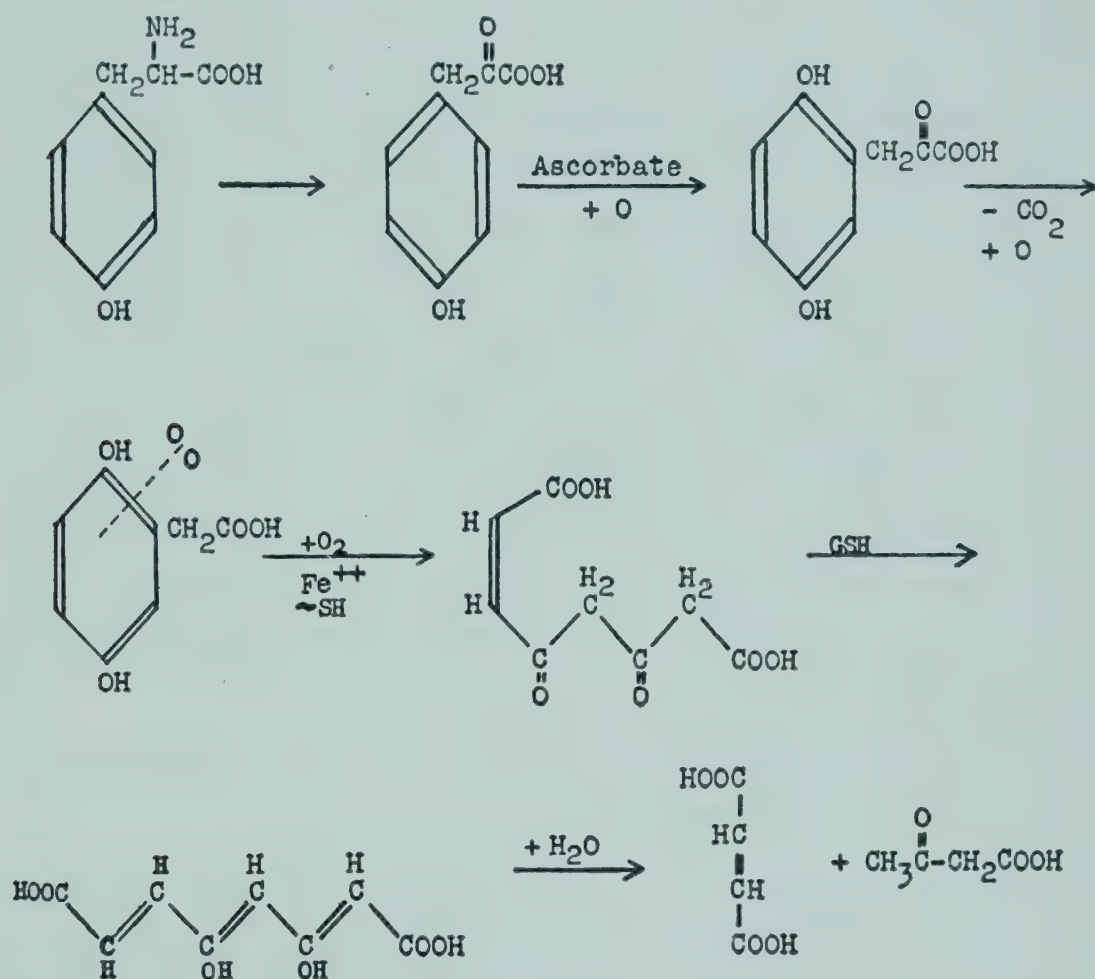


FIG. 18. Diagram of the pathway of tyrosine degradation in mammalian liver. The intermediates following L-tyrosine, are, in order, *p*-hydroxyphenylpyruvic acid, 2,5-dihydroxyphenylpyruvic acid, homogentisic acid, maleyl-acetoacetic acid, fumaryl-acetoacetic acid, and fumaric acid plus acetoacetic acid. (From Knox.)

produced from tyrosine. Deamination of the amino acid is always the preliminary step. Other recent developments concern the steps that follow in the normal condition the formation of homogentisic acid. The immediate product of opening the aromatic ring is maleyl-acetoacetate. This is rearranged into fumarylacetoacetate, and the last compound is hydrolyzed into fumaric acid and acetoacetate. These stages are represented in Fig. 18.

Three particularly interesting aspects of the metabolic pathway to



homogentisic acid concern (1) the transformation of phenylalanine to tyrosine, which in man is blocked in the hereditary condition that is known as phenylketonuria and that in some way produces an extreme mental defect; (2) the requirement for ascorbic acid in the transformation of para-hydroxyphenylpyruvic acid to the dihydroxy acid; and (3) the shifts in position of the side-chains necessary in the same conversion.

The conversion of phenylalanine to tyrosine, by means of an enzyme that it is proposed to call a hydroxylase, is not a reversible reaction. According to Udenfriend and Mitoma, who reported on studies of this enzyme, it is distinct from other hydroxylating enzymes and is specific for phenylalanine. In animals it is found only in the liver, and is a soluble enzyme. Jervis has shown that the livers of two phenylketonuric persons lacked the enzyme. The conversion is actually a two-step process, since two separate enzyme fractions have been obtained by Udenfriend. One enzyme requires DPN or DPNH as coenzyme. The system also requires free oxygen, a simple aldehyde such as benzaldehyde, and ferrous iron. Pyruvate is a strong inhibitor, and yet hydrogen peroxide has not been implicated. The mechanism of the reaction is still very unclear.

Tyrosine transamination specifically requires  $\alpha$ -ketoglutarate, and the usual transaminase cofactor, pyridoxal phosphate. Alanine, a usual byproduct of the over-all tyrosine oxidation reaction, apparently comes from the virtually omnipresent glutamate-alanine transamination system.

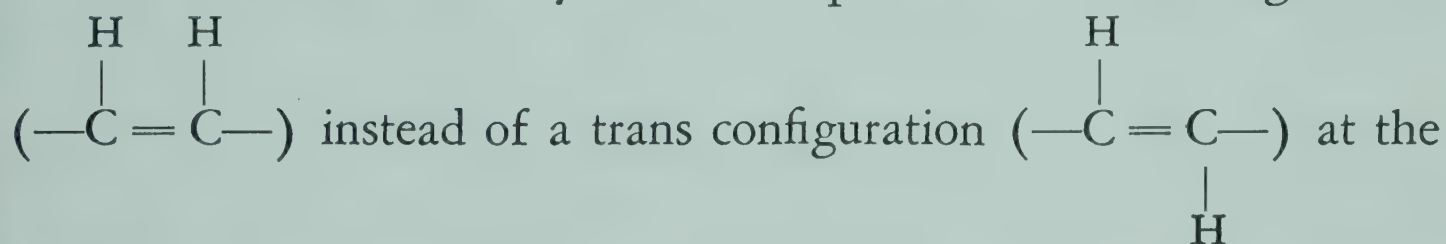
Tyrosine oxidation stops in the absence of ascorbic acid. Knox pointed out the relation of this phenomenon to the vitamin C action of ascorbic acid, and referred to the excretion of parahydroxyphenylpyruvic acid in the urine of scorbutic animals and persons suffering from scurvy. In fact, the system in vitro will provide an assay for the physiological effect of vitamin C.

As to the migration of the side-chains in the oxidation of para-hydroxyphenylpyruvic acid, the discovery of similar migrations, e. g., of methyl groups in the oxidation of para-cresol, has made the change in the present instance from a para-hydroxy to a 2,5-dihydroxy form less surprising. At any rate, when phenylalanine was labeled at



C<sub>1</sub> in the ring and at the  $\alpha$ - and carboxyl-carbon atoms of the side-chain, there were two unlabeled carbon atoms between the labeled ones in the acetoacetate product although there was only one unlabeled carbon in between in the original phenylalanine. This demonstration clearly shows that some shifting of the side-chain must occur during oxidation.

The oxidation of homogentisic acid has been worked out quite fully in the last five years. A most remarkable feature of this reaction is that the enzyme, homogentisate oxidase, requires ferrous iron and yet is not inhibited by carbon monoxide and is presumably therefore not a porphyrin compound. It is however inhibited by the usual sulfhydryl inhibitors and its activity can then be restored by glutathione. It consequently seems to be a new type of enzyme, a true iron-sulfhydryl enzyme like the early models of respiratory enzymes studied by Warburg. The immediate product of the one-step oxidation catalyzed by this enzyme is maleyl-acetoacetate, which differs from the fumaryl-acetoacetate isolated from the system by Ravdin and Crandall only in that it possesses a *cis* configuration



olefinic group. Crandall pointed out that the reaction resembles the oxidations of 3-hydroxyanthranilic acid, catechol, and protocatechuic acid, in that in each of these cases the ring is split across an aromatic carbon-carbon bond, either adjacent to a phenolic group or between two of them. The enzymes which catalyze the oxidations of 3-hydroxyanthranilic acid and catechol are like homogentisic acid oxidase in being reactivated by ferrous ions after various kinds of inhibition. When homogentisic acid oxidase is inactivated by parachloromercuribenzoate, neither glutathione nor ferrous ions alone will reactivate, but both together will do so. This seems to indicate that the iron may be linked to the enzyme by its sulfhydryl groups.

The absorption spectra and bromine titrations of maleyl-acetoacetate and fumaryl-acetoacetate, studied by Knox, indicate that the former exists in acid solutions in the keto form, which because of the



cis configuration of maleyl-acetoacetate would be hook-shaped, and unstable in the enol form; whereas fumaryl-acetoacetate, having a trans configuration, would be linear and have a greater stability in the enol form. An isomerase that converts the cis into the trans form was isolated from the system, and glutathione was found to be the specific cofactor for this conversion also. The hydrolyzing enzyme that splits fumaryl-acetoacetate into fumaric and acetoacetic acids also turned out to be highly specific. Both of the two last steps in this oxidation sequence seem to be irreversible.

### *Tryptophan* (see Fig. 24)

This unique indole-ring amino acid is synthesized in *Neurospora* from indole and serine, pyridoxal phosphate serving as coenzyme for

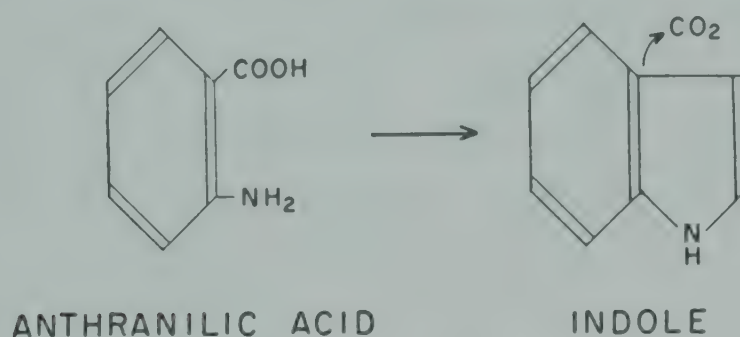


FIG. 19. The conversion of anthranilic acid to indole.  
(After Yanofsky.)

the enzyme, tryptophan desmolase (or synthetase). The precursors of indole are however much less certain. In *E. coli* and *Neurospora* anthranilic acid will satisfy a requirement for indole or tryptophan. There seem to be at least two steps between anthranilic acid and indole. Yanofsky presented evidence that the two newly added carbon atoms in the indole ring arise from glucose and that the completion of the indole ring involves the carbon atom of the aromatic ring to which the carboxyl group of anthranilic acid is attached. Completion of the ring and decarboxylation represent distinct steps (Fig. 19).

Yanofsky also pointed out that anthranilic acid is a product of tryptophan degradation along one pathway, so that its utilization as a precursor implies the existence of a tryptophan-anthranilic-acid cycle (Fig. 20). Haskins and Mitchell have suggested that anthra-

nilic acid is not a direct precursor of indole but is readily converted to and formed from a true intermediate. In *E. coli*, however, there is no evidence for the existence of such a tryptophan cycle. Yanofsky reports that he was unable to detect any conversion of kynurenine to anthranilic acid in that organism, nor of tryptophan to kynurenine.

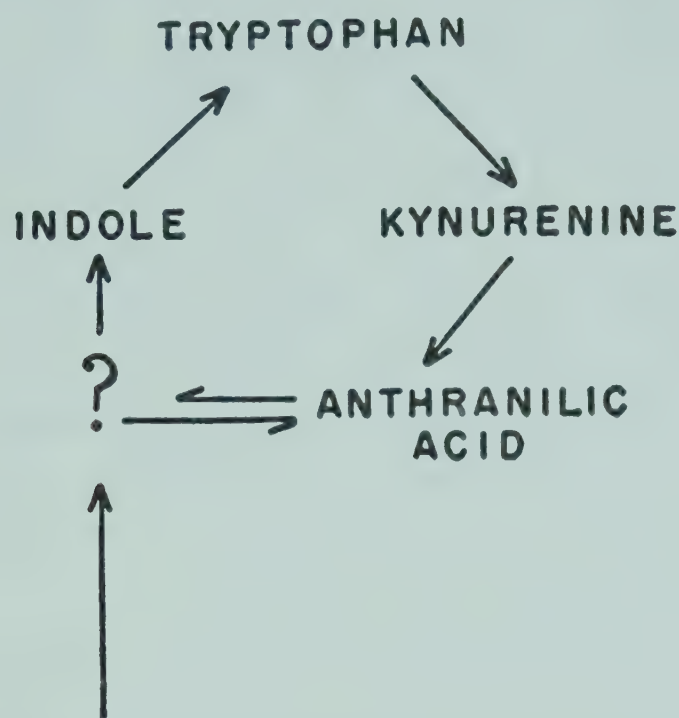


FIG. 20. The tryptophan-anthranilic-acid cycle proposed by Haskins and Mitchell. (From Yanofsky.)

As has already been discussed, shikimic acid is a precursor of indole, but the intervening steps are unknown. In *Bacillus subtilis* mutants blocked between indole and tryptophan do not accumulate indole. In *Lactobacillus arabinosus* the synthesis of tryptophan has been reported not to involve a coupling of indole and serine at all. Thus there seem to be very considerable differences in the synthesis of tryptophan in different species.

Jakoby and Bonner have found that the yield of kynureninase in *Neurospora* is adaptively increased not only by growth on kynurenine but also by growth on kynurenine precursors. Phenylalanine and tyrosine likewise increase the yield of kynureninase, and this observation raises the question whether possibly these other aromatic amino acids are precursors of tryptophan (or kynurenine). Other explanations exist. Thus, if both phenylalanine and tyrosine arise from a common precursor with tryptophan, an extra supply of the two former amino acids might have a sparing action on the utiliza-



tion of the precursor and thus divert more of it into the synthesis of tryptophan. Or phenylalanine and tyrosine might exert a heterologous action on the induction of kynureninase. Speaking against those possibilities is the observation that mutants which require phenylalanine or tyrosine, when supplied with an excess of the respective amino acids, fail to increase the yield of kynureninase, as would be expected whichever of the two proposed explanations held true. Jakoby therefore thinks that the data suggest a conversion of phenylalanine and tyrosine into some precursor of kynurenine. There is also evidence in the contribution by Glass and Plaine that in *Drosophila* tyrosine, and to a smaller extent phenylalanine, exert effects on two suppressor genes that are specifically inhibited in effect by tryptophan. Since animals do not synthesize phenylalanine or tryptophan, there is no possibility of a common precursor in this case. Somehow tyrosine and tryptophan metabolism must be more closely linked than the recognized pathways indicate.

Fig. 21 indicates what is known at the present time of the various paths of tryptophan synthesis and degradation. According to Mehler's comprehensive review, there are five main routes for the degradation of tryptophan. One is its conversion back into indole with pyruvate and ammonia as the other products. This cleavage of tryptophan is carried out by an enzyme called tryptophanase, present in *E. coli* and many other microorganisms, and requiring pyridoxal phosphate as coenzyme. Although the reaction seems to be essentially a reversal of the synthesis of tryptophan from indole and serine, the enzymes are distinct.

A second pathway involves hydroxylation of the indole ring and leads through 5-hydroxytryptophan to 5-hydroxytryptamine (Fig. 22). The latter is also known as serotonin or enteramine, and is a potent vasoconstrictor and blood platelet substance in vertebrates and an invertebrate hormone. Udenfriend and Titus reported some studies on the synthesis of serotonin which seem to exclude tryptamine as a possible intermediate, and indicate that the hydroxylation of the indole ring at position 5 regularly precedes decarboxylation of the side-chain. 5-Hydroxytryptophan was found to occur in toad venom, in which serotonin is converted to the toxin bufotenin by

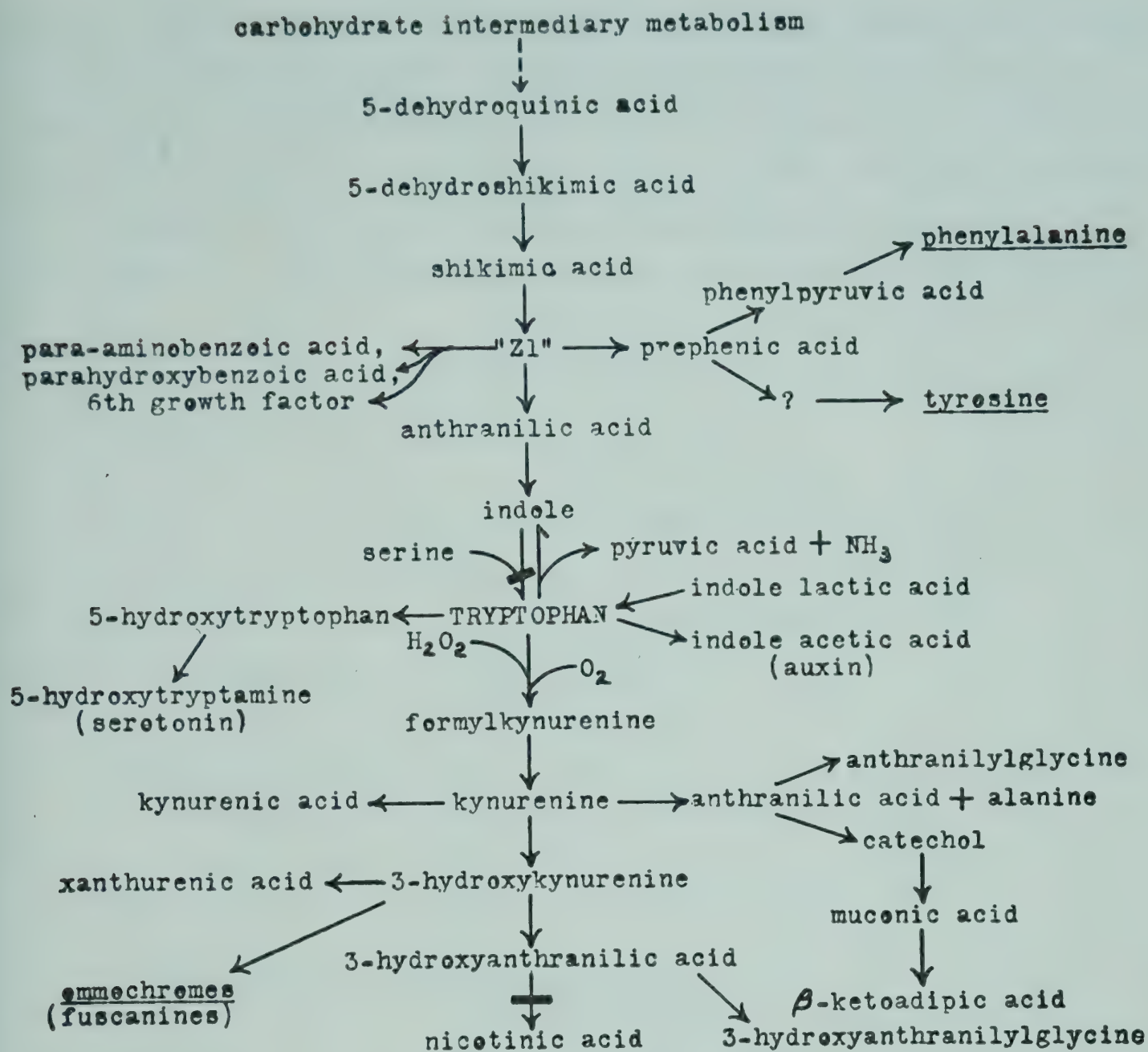


FIG. 21. General scheme of the biosynthesis and degradation of tryptophan. The two blocked reactions bracket the part of tryptophan metabolism characteristic of most animals. (Based on the presentations of Davis and Mehler.)

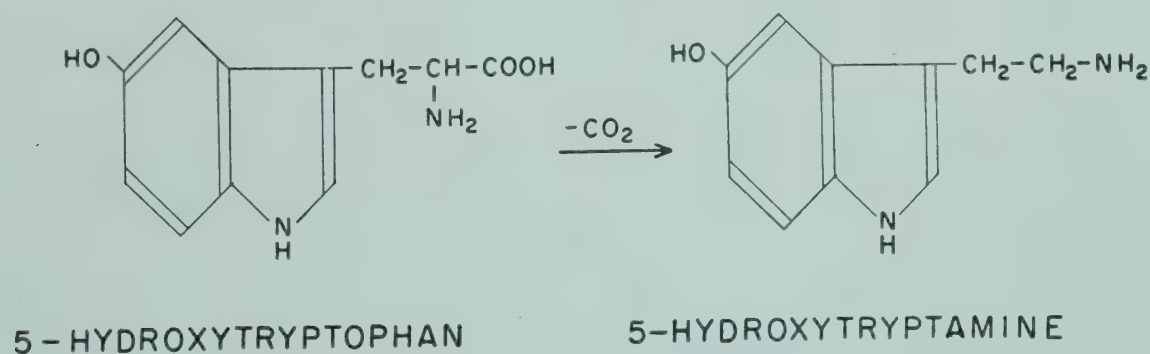


FIG. 22. The formation of serotonin (5-hydroxytryptamine) from 5-hydroxytryptophan.

methylation of the amine. Guinea pig and rat liver slices form 5-hydroxytryptophan from tryptophan, and the specific 5-hydroxy-



tryptophan decarboxylase has been found in many animal tissues. When the hydroxy-amino acid is fed to dogs, it is excreted mainly as 5-hydroxytryptamine. In short, the evidence for the natural occurrence of the new amino acid, 5-hydroxytryptophan, seems quite secure. 5-Hydroxytryptamine itself is metabolized by kidney or liver homogenates, one product, amounting to about 30 per cent of the

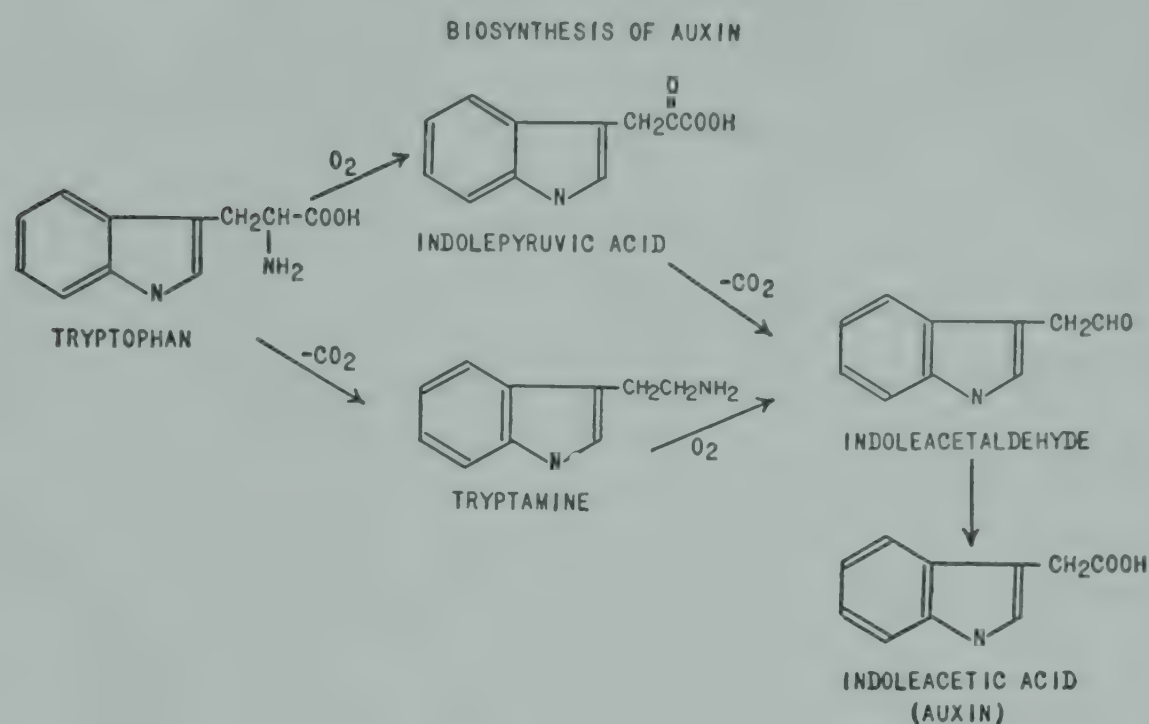


FIG. 23. The synthesis of the auxin indoleacetic acid tryptophan.  
(From Mehler.)

administered drug, being 5-hydroxyindoleacetic acid. Dogs and human beings normally excrete daily in the urine approximately 2 and 7 mg. respectively of the last compound, and the amount greatly increases when either 5-OH-tryptophan or 5-OH-tryptamine is administered, but not when tryptophan itself is given. There is also a rapid metabolism of 5-hydroxytryptamine in rabbits. It may be calculated that about 3 per cent of the dietary tryptophan is metabolized by this route, at least in human beings.

The pathway from tryptophan to indoleacetic acid (Fig. 23), formerly thought to be chiefly of significance in plants because of the importance of indoleacetic acid and other auxins as growth factors, is clearly becoming of significance in animals too. Indoleacetic acid has been isolated from human urine, and the corresponding keto acid must exist in vivo. The immediate precursor of

indoleacetic acid in plants seems to be indoleacetaldehyde, which can be formed either by way of indolepyruvic acid or by way of tryptamine. In the former case the sidechain of the tryptophan molecule is first deaminated and then decarboxylated; in the alternative route the sequence is reversed. It is also possible that by transamination indoleacetaldehyde could be converted to tryptamine by a side reaction. In a pea preparation it has been shown that indoleacetic acid can be converted, by a two-step oxidase-peroxidase reaction, into indolealdehyde.

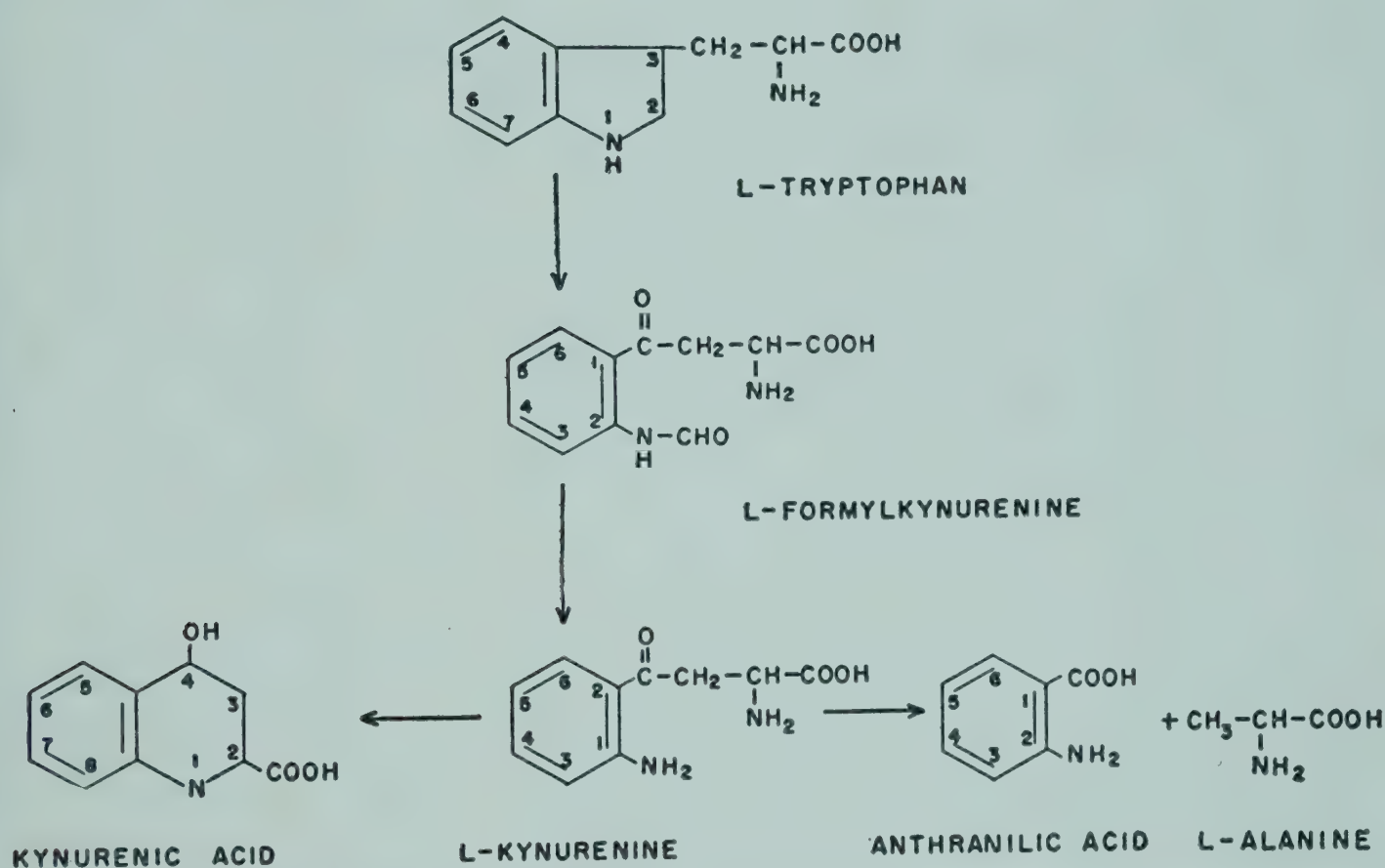


FIG. 24. The degradation of tryptophan to kynurenine and its conversion either to kynurenic acid or to anthranilic acid and alanine. (From Hayaishi.)

Kynurenine has come to occupy a very important position in the scheme of tryptophan metabolism. Knox and Mehler showed that tryptophan is oxidized to kynurenine by a coupled peroxidase-oxidase reaction, the intermediate being formylkynurenine (Fig. 24). The enzyme obtained from mammalian liver is sensitive to carbon monoxide, a fact probably meaning that the iron remains in the ferrous condition, unlike most peroxidases. It is consequently thought that the reaction may be catalyzed by a single enzyme with the ability to act both as oxidase and peroxidase. This enzyme is adaptive, both



in bacteria and mammalian liver. Formylkynurenine is converted to kynurenine by a widespread enzyme (liver, bacteria, *Neurospora*), known as formylase, and specific for L-kynurenine.

Hayaishi finds that the tryptophan peroxidase-oxidase system requires strict specificity of the benzene part of the molecule. Neither 5-hydroxytryptophan nor 7-hydroxytryptophan are appreciably oxidized to the corresponding hydroxykynurenine derivatives, and indeed these compounds competitively inhibit the oxidation of L-tryptophan in the system. The failure of 7-hydroxytryptophan is particularly instructive, since in this instance the hydroxyl group is carried on the benzene ring at the same position as in 3-hydroxykynurenine, the normal oxidative product of kynurenine. It remains unknown how the last-named conversion takes place, but clearly the hydroxylation does not occur before the conversion of tryptophan to kynurenine.

Kynureninase hydrolyzes kynurenine to anthranilic acid and alanine; pyridoxal phosphate is its cofactor (see Fig. 24). The enzyme is widely distributed (mammals, bacteria, *Neurospora*). Bacterial (*Pseudomonas*) kynureninase, according to Hayaishi's studies, is less specific than the tryptophan oxidase-peroxidase, for it acts on 3-hydroxykynurenine and 5-hydroxykynurenine as well as on kynurenine itself (activity ratio 2:5:10). It does not act on formylkynurenine, although the corresponding enzyme from *Neurospora* does so. The aromatic amino group must remain free and unaltered in position for full activity to be retained.

Kynurenine is also converted, by means of a transamination, into the homologous keto acid, which undergoes ring closure, so as to form kynurenic acid. The same *Neurospora* transaminase also changes 3-hydroxykynurenine into xanthurenic acid; and Hayaishi finds that the *Pseudomonas* kynurenine transaminase acts analogously with little specificity on 5-hydroxykynurenine as well, the product in this case being a new compound, 6-hydroxykynurenic acid. The ratio of the activities on the three substrates was 10:8:4, respectively.

Glass and Plaine contributed an account of the relation of some of the above steps in tryptophan metabolism to the action of two suppressor genes in *Drosophila*, both of which inhibit forms of abnormal growth, one being in the eyes, the other in the form of



melanotic tumors in the body cavity. An excess of tryptophan, over and above the amount in the normal diet, inhibits both suppressor genes and allows the two suppressed mutants to manifest themselves in a high proportion of individuals. X-ray treatment of embryonic or larval individuals produces a similar effect, and both the x-ray effect and the tryptophan effect are potentiated by oxygen and are inhibited by cysteine. The site of this action is thought to be the tryptophan oxidase-peroxidase reaction, and the action of the suppressor genes is interpreted as being that of raising the threshold of response to tryptophan or some oxidized derivative of it above the level of the mutant types and near to that of the wild-type. Especially striking is the fact that the suppressor of the tumor gene is inhibited by anthranilic acid and by indole as much as by tryptophan, whereas these compounds have a much smaller effect on the suppressor of erupt eyes. On the other hand, serine has no effect on the suppressor of melanotic tumors but a significant effect on the suppressor of erupt eyes. When indole and serine are fed together, there seems to be a mutual interaction that leads to a more nearly normal balance indicated by the reduction in both types of abnormal growth.

3-Hydroxykynurenine gives rise to various insect pigments. It is also converted to 3-hydroxyanthranilic acid through the kynureninase reaction, and in *Neurospora* this pathway leads, perhaps through quinolinic acid, to nicotinic acid. In the rat, there appears to be no essential difference in the route. In bacteria, according to studies of *E. coli* and *B. subtilis* by Yanofsky, kynureninase is lacking, and 3-hydroxyanthranilic acid therefore cannot be an intermediate in the synthesis of nicotinic acid. In fact, isotopic tracers revealed that neither indole nor tryptophan could be a major precursor of nicotinic acid in these organisms. (It is interesting that once again, as so often during the symposium, the greater similitude between *Neurospora* and mammals than between *E. coli* and mammals was brought out in the present instance.)

A final pathway of tryptophan degradation described by Mehler was what he termed the "aromatic pathway" (Fig. 25). This is utilized by certain bacteria (viz., *Pseudomonas*) that make use of



tryptophan as a major source of energy. Branching from kynurenine to anthranilic acid and alanine (the kynureninase reaction), this oxidative pathway leads through catechol, *cis, cis*-muconic acid, and  $\beta$ -ketoadipic acid in successive steps. The enzyme pyrocatechase, which produces catechol, is adaptive, and has been isolated by Japanese workers. Like so many other enzymes in the metabolism of the aromatic amino acids, it requires ferrous iron as a cofactor.

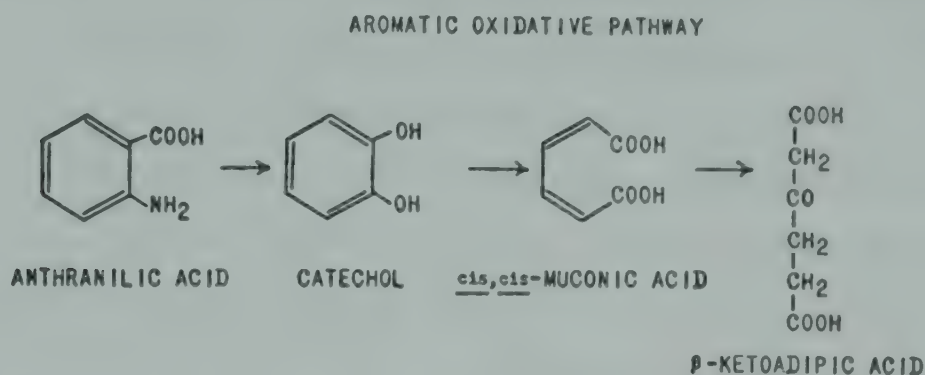


FIG. 25. The degradation of tryptophan via anthranilic acid to  $\beta$ -ketoadipic acid in bacteria such as *Pseudomonas* which use tryptophan for energy. (From Mehler.)

### CONCLUSION

Undoubtedly the outstanding feature of the recent advances in the understanding of amino acid metabolism presented in the current symposium has been that of the expanding interrelationships of the several groups of amino acids. Although the treatment has necessarily been that of considering together in separate sessions of the symposium and in separate parts of the book those amino acids that are clearly most related to one another, one could now draw up a single great metabolic scheme in which all of them were interconnected. The numerous transaminations which have received emphasis from the beginning of the symposium to the end, and the amino acid pool to which at least sixteen amino acids contribute, form only a part of those interrelationships. Glutamic and aspartic acids stand at a crossroad from which there radiate the connections between the tricarboxylic acid cycle and the ornithine-citrulline-arginine cycle; and the proline-hydroxyproline pathway projects from glutamic acid in yet another direction. Histidine connects on the one hand with pentose metabolism, and on the other, through the

donation of the active one-carbon unit it forms during its own demise, it participates in the synthesis of the nucleotides and of the new methyl groups needed in making methionine, choline, etc.; while in a third connection it ties up with glutamic acid. Valine, leucine, and isoleucine are synthesized by parallel and interconnecting paths and through their degradation terminate in acetoacetate, fatty acid metabolism and the Krebs cycle. The synthesis of valine and isoleucine ties them to threonine and homoserine, and through the latter to the vast complex of the methionine-homocysteine-cystathionine-cysteine-homoserine group. Lysine metabolism debouches once again at the glutamic acid nodal point, and in bacteria connects with the aspartic-acid-homoserine-threonine-methionine complex. Glycine and serine unite the metabolism of glyoxylic acid, glycolic acid, formic acid, and formaldehyde with the methionine-choline-betaine-sarcosine cycle, with the synthesis of the porphyrins, and with purine synthesis. The aromatic amino acids trace back to the carbohydrates, especially to sedoheptulose diphosphate, through shikimic acid; and in their degradative metabolism they are reassembled into pigments, hormones, auxins, and vitamins. The alanine side-chains of phenylalanine, tyrosine, and tryptophan reveal yet another relationship, and the degradation of phenylalanine and tyrosine into fumaric acid flows once again into the Krebs cycle; while tryptophan is significantly connected with nicotinic acid and thereby with the pyridine nucleotides.

Nor should one neglect to mention, in final appraisal, the discovery of a considerable number of new naturally occurring amino acids and the deeper insight obtained into the mechanism of action of various coenzymes, in particular of the folic acid derivatives. Not least interesting is the discovery of the long-anticipated ferrous-sulfhydryl type of enzyme.

If the visualization of amino acid metabolism is at length coming into focus, this does not mean that all the unknown areas have by any means been explored. In a measure biochemists stand today in a position like the seamen and geographers of the seventeenth century. The coasts of the great continents and islands were then known, but the interiors of the great new worlds of Africa, America



—North and South—and Australia were empty blanks decoratively ascribed by cartographers to real and imaginary monsters. Some of today's theories of amino acid relationships, enzyme and coenzyme mechanisms, and maybe the supposed parallels between *Neurospora* and man, to say nothing of *E. coli*, may be fated to evaporate in the light of cold knowledge, like the sea-serpent, or to be deflated, like the mysterious behemoth, into the ordinary and ugly hippopotamus. But though no one will reach El Dorado or the Seven Cities of Cibola, there is still a Mexico, Cuzco, Timbuctoo, and Kalgoorlie for the intrepid treasure-seeker. In the intricacies of amino acid metabolism there is a continent yet to be explored.

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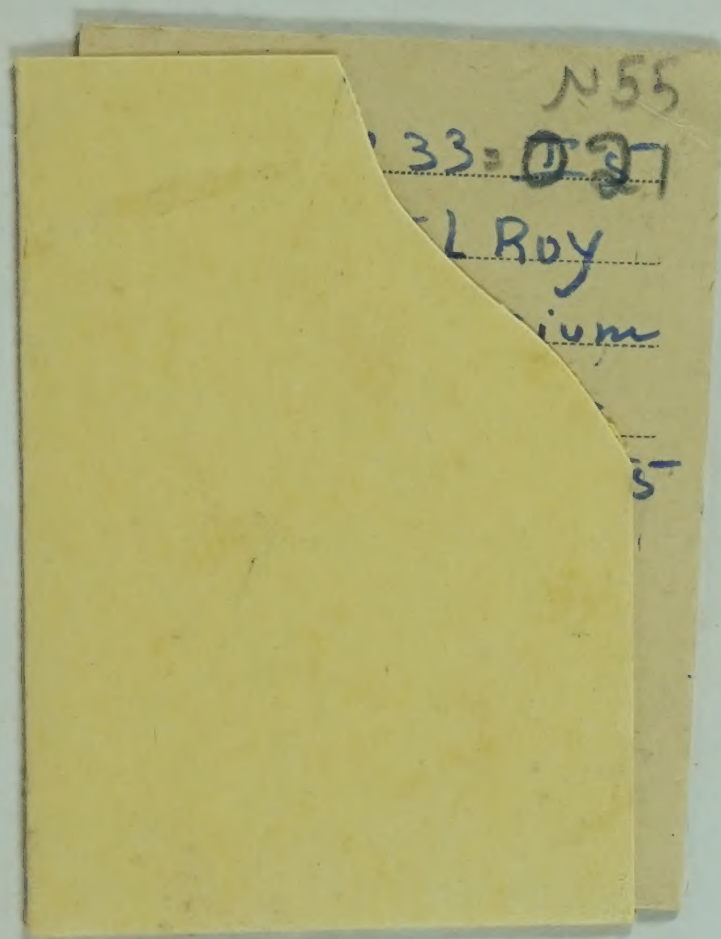












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